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March 11, 1997

***Box: New Patent Application***

Assistant Commissioner for Patents
Washington, D.C. 20231

Re: U.S. Non-Provisional Utility Patent Application
Appl. No. To be assigned; Filed: March 11, 1997
For: **Method for the Identification of Agents for Use in the Treatment of Alzheimer's Disease**
Inventors: Atwood *et al.*
Our Ref: 0609.4350000/REF/LLK

Sir:

The following documents are forwarded herewith for appropriate action by the U.S. Patent and Trademark Office:

1. U.S. Non-Provisional Utility Patent Application entitled:

Method for the Identification of Agents for Use in the Treatment of Alzheimer's Disease

and naming as inventors:

Craig S. Atwood et al
Ashley I. Bush
Xudong Huang, and
Rudolph E. Tanzi

the application comprising:

a. A specification containing:

(i) 49 pages of description prior to the claims;

(ii) 9 pages of claims (39 claims);

(iii) a one (1) page abstract;

b. 17 sheets of drawings: (Figures 1-12); and

2. Two (2) return postcards.

It is respectfully requested that, of the two attached postcards, one be stamped with the filing date of these documents and returned to our courier, and the other, prepaid postcard, be stamped with the filing date and unofficial application number and returned as soon as possible.

This patent application is being submitted under 37 C.F.R. § 1.53(b)(1) without Declaration and without filing fee.

Respectfully submitted,

STERNE, KESSLER, GOLDSTEIN & FOX P.L.L.C.

Lori L. Kerber
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REF/LLK:ttp
Enclosures

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# Method for the Identification of Agents for Use in the Treatment of Alzheimer's Disease

***Statement as to Rights to Inventions Made Under Federally-Sponsored Research and Development***

Part of the work performed during the development of this invention utilized U.S. Government Funds under Grant No. R29AG12686 from the National Institutes of Health. The government may have certain rights in this invention.

## *Background of the Invention*

Polymers of Abeta (Aβ), the 4.3 kD, 39-43 amino acid peptide product of the transmembrane protein, amyloid protein precursor (APP), are the main components extracted from the neuritic and vascular amyloid of Alzheimer's disease (AD) brains. Aβ deposits are usually most concentrated in regions of high neuronal cell death, and may be present in various morphologies, including amorphous deposits, neurophil plaque amyloid, and amyloid congophilic angiopathy (Masters, C.L., *et al., EMBO J. 4*:2757 (1985); Masters, C.L. *et al., Proc. Natl. Acad. Sci. USA 82*: 4245 (1985)). Growing evidence suggests that amyloid deposits are intimately associated with the neuronal demise that leads to dementia in the disorder.

The presence of an enrichment of the 42 residue species of Aβ in these deposits suggests that this species is more pathogenic. The 42 residue form of Aβ ($A\beta_{1-42}$), while a minor component of biological fluids, is highly enriched in amyloid, and genetic studies strongly implicate this protein in the etiopathogenesis of AD. Amyloid deposits are decorated with inflammatory response proteins, but biochemical markers of severe oxidative stress such as peroxidation adducts, advanced glycation end-products, and protein cross-linking are seen in proximity to the lesions. To date, the cause of Aβ deposits is unknown, although it is believed that preventing these deposits may be a means of treating the disorder.

When polymers of Aβ are placed into culture with rat hippocampal neurons, they are neurotoxic (Kuo, Y-M., *et al., J. Biol. Chem. 271*:4077-81 (1996); Roher, A.E., *et al., Journal of Biological Chemistry 271*:20631-20635 (1996)). The mechanism underlying the formation of these neurotoxic polymeric Aβ species remains unresolved. The overexpression of Aβ alone cannot sufficiently explain amyloid formation, since the concentration of Aβ required for precipitation is not physiologically plausible. That alterations in the neurochemical environment are required for amyloid formation is indicated by its solubility in neural phosphate buffer at concentrations of up to 16 mg/ml (Tomski, S. & Murphy, R.M. *Archives of Biochemistry and Biophysics 294*:630 (1992)), biological fluids such as cerebrospinal fluid (CSF) (Shoji, M., Golde *et al.* (1992); Seubert, P. (1992); Haass *et al.* (1992)) and in the plaque-free brains of Down's syndrome patients (Teller, J.K., *et al., Nature Medicine 2*:93-95 (1996)).

Studies into the neurochemical vulnerability of Aβ to form amyloid have suggested altered zinc and $[H^+]$ homeostasis as the most likely explanations for amyloid deposition. Aβ is rapidly precipitated under mildly acidic conditions *in vitro* (pH 3.5-6.5) (Barrow C.J. & Zagorski M.G., *Science 253*:179-182 (1991); Fraser, P.E., *et al., Biophys. J. 60*:1190-1201 (1991); Barrow, C.J., *et al., J. Mol. Biol. 225*:1075-1093 (1992); Burdick, D., *J. Biol. Chem.* 267:546-554 (1992); Zagorski, M.G. and Barrow, C.J., *Biochemistry 31*:5621-5631 (1992); Kirshenbaum, K. and Daggett, V., *Biochemistry 34*:7629-7639 (1995); Wood, S.J., *et al., J. Mol. Biol. 256*:870-877 (1996)). Recently, it has been shown that the presence of certain biometals, in particular redox inactive $Zn^{2+}$ and, to a lesser extent, redox active $Cu^{2+}$ and $Fe^{2+}$, markedly increases the precipitation of soluble Aβ (A.T. Bush *et al., J. Biol. Chem. 268*:16109 (1993); A.I. Bush *et al., J. Biol. Chem. 269*:12152 (1994); A.I. Bush *et al., Science 265*:1464 (1994); A.I. Bush, *et al., Science 268*:1921 (1995)). At physiological pH, $A\beta_{1-40}$ specifically and saturably binds $Zn^{2+}$, manifesting high affinity binding ($K_D$ = 107 nM) with a 1:1 ($Zn^{2+}$:Aβ) stoichiometry, and low affinity binding ($K_D$ = 5.2 μM) with a 2:1

stoichiometry. However, the complete reversibility of Zn-induced $A\beta_{1-40}$ aggregation in the presence of divalent metal ion chelating agents suggests that zinc binding is a reversible, normal function of Aβ and implicates other neurochemical mechanisms in the formation of amyloid. A process involving irreversible Aβ aggregation, such as the crosslinking of Aβ monomers, in the formation of polymeric species of Aβ that are present in amyloid plaques is thus a more plausible explanation for the formation of neurotoxic polymeric Aβ species.

The reduction by APP of copper (II) to copper (I) may lead to irreversible Aβ aggregation and crosslinking. This reaction may promote an environment that would enhance the production of hydroxyl radicals, which may contribute to oxidative stress in AD (Multhaup, G., *et al., Science 271*:1406-1409 (1996)). A precedence for abnormal Cu metabolism already exists in the neurodegenerative disorders of Wilson's disease, Menkes' syndrome and possibly familial amyotrophic lateral sclerosis (Tanzi, R.E. *et al., Nature Genetics 5*:344 (1993); Bull, P.C., *et al., Nature Genetics 5*:327-x (1993); Vulpe, C., *et al., Nature Genetics 3*:7 (1993); Yamaguchi, Y., *et al., Biochem. Biophys. Res. Commun. 197*:271 (1993); Chelly, J. *et al., Nature Genetics 3*:14 (1993); Wang, D. & Munoz, D.G., *J. Neuropathol. Exp. Neurol. 54*:548 (1995); Beckman, J.S., *et al., Nature 364*:584 (1993); Hartmann, H.A. & Evenson, M.A., *Med. Hypotheses 38*:75 (1992)).

Although much fundamental pathology, genetic susceptibility and biology associated with AD is becoming clearer, a rational chemical and structural basis for developing effective drugs to prevent or cure the disease remains elusive. While the genetics of the disorder indicates that the metabolism of Aβ is intimately associated with the etiopatholgenesis of the disease, drugs for the treatment of AD have so far focused on "cognition enhancers" which do not address the underlying disease processes.

## *Summary of the Invention*

The invention relates to a method for the identification of an agent to be used in the treatment of AD, wherein the agent is capable of altering the production of Cu(I) by Aβ, the method comprising:

(a) adding Cu(II) to a first Aβ sample;

(b) allowing the first sample to incubate for an amount of time sufficient to allow said first sample to generate Cu(I);

(c) adding Cu(II) to a second Aβ sample, the second sample additionally comprising a candidate pharmacological agent;

(d) allowing the second sample to incubate for the same amount of time as the first sample;

(e) determining the amount of Cu(I) produced by the first sample and the second sample; and

(f) comparing the amount of Cu(I) produced by the first sample to the amount of Cu(I) produced by the second sample;

whereby a difference in the amount of Cu(I) produced by the first sample as compared to the second sample indicates that the candidate pharmacological agent has altered the production of Cu(I) by Aβ.

In a preferred embodiment, the amount of Cu(I) present in said first and said second sample is determined by

(a) adding a complexing agent to said first and said second sample, wherein said complexing agent is capable of combining with Cu(I) to form a complex compound, wherein said complex compound has an optimal visible absorption wavelength;

(b) measuring the absorbancy of said first and said second sample; and

(c) calculating the concentration of Cu(I) in said first and said second sample using the absorbancy obtained in step (b).

In a more preferred embodiment, the complexing agent is bathocuproinedisulfonic (BC) anion. The concentration of $Cu^{+}$ produced by Aβ may then be calculated on the basis of the absorbance of the sample at about 478 nm to about 488 nm, more preferable about 480 to about 486 nm, and most preferably about 483 nm.

In another aspect, the invention relates to a method for the identification of an agent to be used in the treatment of AD, wherein said agent is capable of altering the production of Fe(II) by Aβ, said method comprising:

(a) adding Fe(III) to a first Aβ sample;

(b) allowing said first sample to incubate for an amount of time sufficient to allow said first sample to generate $Cu^{+}$;

(c) adding Fe(III) to a second Aβ sample, said second sample additionally comprising a candidate pharmacological agent;

(d) allowing said second sample to incubate for the same amount of time as said first sample;

(e) determining the amount of Fe(II) produced by said first sample and said second sample; and

(f) comparing the amount of Fe(II) present in said first sample to the amount of Fe(II) present in said second sample;

whereby a difference in the amount of Fe(II) present in said first sample as compared to said second sample indicates that said candidate pharmacological agent has altered the production of Fe(II) by Aβ.

In a preferred embodiment, the amount of Fe(II) present is determined by using a spectrophotometric method analagous to that used for the determination of Cu(I), above. In this method, the complexing agent is batho-phenanthrolinedisulfonic (BP) anion. The concentration of $Fe^{2+}$-BP produced by Aβ may then be calculated on the basis of the absorbance of the sample at about 530 to about 540 nm, more preferably about 533 nm to about 538 nm, and most preferably about 535 nm.

In yet another aspect, the invention relates to a method for the identification of an agent to be used in the treatment of AD, wherein said agent is capable of altering the production of $H_2O_2$ by Aβ, said method comprising:

(a) adding Cu(II) or Fe(III) to a first Aβ sample;

(b) allowing said first sample to incubate for an amount of time sufficient to allow said first sample to generate $H_2O_2$;

(c) adding Cu(II) or Fe(III) to a second Aβ sample, said second sample additionally comprising a candidate pharmacological agent;

(d) allowing said second sample to incubate for the same amount of time as said first sample;

(e) determining the amount of $H_2O_2$ produced by said first sample and said second sample; and

(f) comparing the amount of $H_2O_2$ present in said first sample to the amount of $H_2O_2$ present in said second sample;

whereby a difference in the amount of $H_2O_2$ present in said first sample as compared to said second sample indicates that said candidate pharmacological agent has altered the production of $H_2O_2$ by Aβ.

In a preferred embodiment, the determination of the amount of $H_2O_2$ present in said first and said second sample is determined by

(a) adding catalase to a first aliquot of said first sample obtained in step (a) of claim 1 in an amount sufficient to break down all of the $H_2O_2$ generated by said sample;

(b) adding TCEP, in an amount sufficient to capture all of the $H_2O_2$ present in said samples, to

(i) said first aliquot

(ii) a second aliquot of said first sample obtained in step (a) of claim 1; and

(iii) said second sample obtained in step (b) of claim 1;

(c) incubating the samples obtained in step (b) for an amount of time sufficient to allow the TCEP to capture all of the $H_20_2$;

(d) adding DTNB to said samples obtained in step (c);

(e) incubating said samples obtained in step (d) for an amount of time sufficient to generate TMB;

(f) measuring the absorbancy at about 407to about 417 nm of said samples obtained in step (e); and

(g) calculating the concentration of $H_2O_2$ in said first and said second sample using the absorbancies obtained in step (f). In a preferred embodiment, the absorbancy of TMB is measured at about 412 nm.

In another aspect, the invention relates to a method for the identification of an agent to be used in the treatment of AD, wherein said agent is capable of decreasing the production of $O_2$- by Aβ, said method comprising:

(a) adding Aβ and to a first buffer sample having an $O_2$ tension greater than 0;

(b) allowing said first sample to incubate for an amount of time sufficient to allow said first sample to generate $O_2$-;

(c) adding Aβ and a candidate pharmacological agent to a second buffer sample having an $O_2$ tension greater than 0;

(d) allowing said second sample to incubate for the same amount of time as said first sample;

(e) determining the amount of $O_2$- produced by said first sample and said second sample; and

(f) comparing the amount of $O_2$- present in said first sample to the amount of $O_2$- present in said second sample;

whereby a difference in the amount of $O_2$- present in said first sample as compared to said second sample indicates that said candidate pharmacological agent has altered the production of $O_2$- by Aβ. In a preferred embodiment, the Aβ used is $A\beta_{1-42}$.

Because the ability of Aβ to generate $H_2O_2$ from $O_2$- may in many instances be beneficial, in a prefered embodiment, the invention also relates to a method for the identification of an agent to be used in the treatment of AD,

wherein said agent is capable of interfering with the interaction of $O_2$ and Aβ to produce $O_2$-, without interfering with the SOD-like activity of Aβ, said method comprising:

(a) identifying an agent capable of decreasing the production of $O_2$- by Aβ; and

(b) determining the ability of said agent to alter the SOD-like activity of Aβ. In a preferred embodiment, the determination of the ability of said agent to alter the SOD-like activity of Aβ is made by determining whether Aβ is capable of catalytically producing Cu(I), Fe(II) or $H_2O_2$.

## *Brief Description of the Figures*

Figure 1 is a graph showing the proportion of soluble $A\beta_{1-40}$ remaining following centrifugation of reaction mixtures.

Figures 2A, 2B and 2C: Figure 2A is a graph showing the proportion of soluble $A\beta_{1-40}$ remaining in the supernatant after incubation with various metal ions. Figure 2B is a graph showing a turbidometric analysis of pH effect on metal ion-induced $A\beta_{1-40}$ aggregation. Figure 2C is a graph showing the proportion of soluble $A\beta_{1-40}$ remaining in the supernatant after incubation with various metal ions, where high metal ion concentrations were used.

Figure 3 is a graph showing a competition analysis of $A\beta_{1-40}$. binding to $Cu^{2+}$.

Figures 4A, 4B and 4C: Figure 4A is a graph showing the proprtion of soluble $A\beta_{1-40}$ remaining in the supernatant following incubation at various pHs in PBS ± $Zn^{2+}$. Figure 4B is a graph showing the proprtion of soluble $A\beta_{1-40}$ remaining in the supernatant following incubation at various pHs with different $Cu^{2+}$ concentrations. Figure 4C is a graph showing the relative aggregation ofnM concentrations of $A\beta_{1-40}$ at pH 7.4 and 6.6 with different $Cu^{2+}$ concentrations.

Figures 5A and 5B: Figure 5A is a graph showing a turbidometric analysis of $Cu^{2+}$-induced $A\beta_{1-40}$ aggregation at pH 7.4 reversed by successive cycles of

chelator. Figure 5B is a graph showing a turbidometric analysis of the reversibility of $Cu^{2+}$-induced $A\beta_{1-40}$ aggregation as the pH cycles between 7.4 and 6.6.

Figure 6 shows the amino acid sequence of $APP_{669-716}$ near $A\beta_{1-42}$. Rat Aβ is mutated (R5G, Y10F, H13R; bold). Possible metal-binding residues are underlined.

Figure 7 is a graph showing the effects of pH, $Zn^{2+}$ or $Cu^{2+}$ upon Aβ formation.

Figure 8 is a western blot showing the extraction of Aβ from post-mortem brain tissue.

Figure 9 is a western blot showing Aβ crosslinking by copper.

Figure 10 is a graph showing Cu(I) generation by Aβ.

Figure 11 is a graph showing $H_2O_2$ production by Aβ.

Figure 12 is a model for free radical and amyloid formation in Alzheimer's disease.

## *Detailed Description of the Preferred Embodiments*

### *Definitions*

In the description that follows, a number of terms are utilized extensively. In order to provide a clear and consistent understanding of the specification and claims, including the scope to be given such terms, the following definitions are provided.

**Aβ peptide** is also known in the art as Aβ, β protein, β-A4 and A4. In the present invention, the Aβ peptide may be comprised of peptides $A\beta_{1-39}$, $A\beta_{1-40}$, $A\beta_{1-41}$, $A\beta_{1-42}$, and $A\beta_{1-43}$. The most preferred embodiment of the invention makes use of $A\beta_{1-40}$. However, any of the Aβ peptides may be employed according to the present invention. The sequence of Aβ peptide is found in C. Hilbich *et al.*, *J. Mol. Biol. 228*:460-473 (1992).

**Amyloid** as is commonly known in the art, and as is intended in the present specification, is a form of aggregated protein.

**Aβ Amyloid** is an aggregated Aβ peptide. It is found in the brains of patients afflicted with AD and DS and may accumulate following head injuries.

**Physiological solution** as used in the present specification means a solution which comprises compounds at physiological pH, about 7.4, which closely represents a bodily or biological fluid, such as CSF, blood, plasma, et cetera.

**Zinc**, unless otherwise indicated, means salts of zinc, i.e., $Zn^{2+}$ in any form, soluble or insoluble.

**Copper(II)**, unless otherwise indicated, means salts of Cu(II), i.e., $Cu^{2+}$ in any form, soluble or insoluble.

**Copper(I)**, unless otherwise indicated, means salts of Cu(I), i.e., $Cu^{+}$ in any form, soluble or insoluble.

**Biological fluid** means fluid obtained from a person or animal which is produced by said person or animal. Examples of biological fluids include but are not limited to cerebrospinal fluid (CSF), blood, serum, and plasma. In the present invention, biological fluid includes whole or any fraction of such fluids derived by purification by any means, *e.g.*, by ultrafiltration or chromatography.

The aim of the present invention is to clarify both the factors which contribute to the neurotoxicity of Aβ polymers and the mechanism which underlies their formation. These findings can then be used to (i) identify agents that can be used to decrease the neurotoxicity of Aβ, as well as the formation of Aβ polymers, and (ii) utilize such agents to develop methods of preventing, treating or alleviating the symptoms of AD.

The present invention relates to the unexpected discovery that Aβ peptides directly produce oxidative stress through the generation of abundant reactive oxygen species (ROS), which include hydroxyl radical (OH·) and hydrogen peroxide ($H_2O_2$). The production of ROS occurs by a metal (Cu, Fe) dependant,

pH mediated mechanism, wherein the reduction of Cu(II) to Cu(I), or Fe(III) to Fe(II), is catalyzed by Aβ. Aβ is highly efficient at reducing Cu(II) and Fe(III).

All the redox properties of $A\beta_{1-40}$ (the most abundant form of soluble Aβ) are exaggerated in $A\beta_{1-42}$. Additionally, $A\beta_{1-42}$, but not $A\beta_{1-40}$, recruits $O_2$ into spontaneous generation of another ROS, $O_2$-, which also occurs in a metal-dependent manner. The exaggerated redox activity of $A\beta_{1-42}$ and its enhanced ability to generate ROS are likely to be the explanation for its neurotoxic properties. Interestingly, the rat homologue of Aβ, which has 3 substitutions that have been shown to attenuate zinc binding and zinc-mediated precipitation. The rat homologue also exhibits less redox activity than its human counterpart. This may explain why the rat is exceptional in that it is the only mammal that does not exhibit amyloid pathology with age. All other mammals analyzed to date possess the human Aβ sequence.

The sequence of ROS generation by Aβ follows the pathway of superoxide-dismutation, which leads to hydrogen peroxide production in a Cu/Fe-dependent manner. After forming $H_2O_2$, the hydroxyl radical (OH·) is rapidly formed by a Fenton reaction with the Fe or Cu that is present, even when these metals are only at trace concentrations. The OH· radical is very reactive and rapidly attacks the Aβ peptide, causing it to cross-link and polymerize. This is very likely to be the chemical mechanism that causes the covalent cross-linking that is seen in mature plaque amyloid. Importantly, the redox activity of Aβ is not attenuated by precipitation of the peptide, suggesting that, *in vivo*, amyloid deposits could be capable of generating ROS *in situ* on an enduring basis. This suggests that the major source of the oxidative stress in an AD-affected brain are amyloid deposits.

A model for free radical and amyloid formation in AD is shown in Fig. 12. The proposed mechnism is explained as follows.

(1) Soluble and precipitated Aβ species possess superoxide dismutase (SOD)-like activity. Superoxide ($O_2^-$), the substrate for the dismutation, is generated both by spillover from mitochondrial respiratory metabolism, and by

$A\beta_{1-42}$ itself (see FIG. 11). Aβ-mediated dismutation produces hydrogen peroxide ($H_2O_2$), requiring Cu(II) or Fe(III), which are reducted during the reaction. Since $H^+$ is required for $H_2O_2$ production, an acidotic environment will increase the reaction.

(2) $H_2O_2$ is relatively stable, and freely permeable across cell membranes. Normally, it will be broken down by intercellular catalase or glutathione peroxidase.

(3) In aging and AD, levels of $H_2O_2$ are high, and catalase and peroxidase activities are low. If $H_2O_2$ is not completely catalyzed, it will react with reduced Cu(I) and Fe(II) in the vicinity of Aβ to generate the highly reduced Cu(I) and Fe(II) in the vicinity of Aβ to generate the highly reactive hydroxyl radical (OH•) by Fenton chemistry.

(4) OH• engenders a non-specific stress and inflammatory response in local tissue. Among the neurochemicals that are released from microglia and possibly neurons in the response are Zn(II), Cu(II) and soluble Aβ. Familial AD increases the likelihood that $A\beta_{1-42}$ will be released at this point. Local acidosis is also part of the stress/inflammatory response. These factors combine to make Aβ precipitate and accumulate, presumably so that it may function *in situ* as an SOD, since these factors induce reversible polymerization. Hence, more soluble Aβ species decorate the perimeter of the accumulating plaque deposits.

(5) If Aβ encounters OH•, it will covalently cross-link during the oligomerization process, making it a more difficult accumulation to resolubilize, and leading to the formation of SDS-resistant oligomers characteristic of plaque amyloid.

(6) If $A\beta_{1-42}$ accumulates, it has the property of recruiting $O_2$ as a substrate for the abundant production of $O_2^-$ by a process that is still not understood. Since $O_2$ is abundant in the brain, $A\beta_{1-42}$ is responsible for setting off a vicious cycle in which the accumulation of covalently linked Aβ is a product of the unusual ability of Aβ to reduce $O_2$, and feed an abundant substrate ($O_2^-$) to itself for dismutation, leading to OH• formation. The production of abundant free

radicals by the accumulating amyloid may further damage many systems including metal regulatory proteins, thus compounding the problem. This suggests that the major source of the oxidative stress in an AD-affected brain are amyloid deposits.

The metal-dependent chemistry of Aβ-mediated superoxide dismutation is reminiscent of the activity of superoxide dismutase (SOD). Interestingly, mutations of SOD cause amyotrophic lateral sclerosis, another neurodegenerative disorder. SOD is predominantly intracellular, whereas Aβ is constitutively found in the extracellular spaces where it accumulates. Investigation of Aβ by laser flash photolysis confirmed the peptide's SOD-like activity, suggesting that Aβ may be an anti-oxidant under physiological circumstances. Since $H_2O_2$ has been shown to induce the production of Aβ, the accumulation of Aβ in AD may reflect a response to an oxidant stress paradoxically caused by Aβ excess. This may cause and, in turn, be compounded by, damage to the biometal homeostatic mechanisms in the brain environment.

Thus, it has recently been discovered (i) that much of the Aβ aggregate in AD-affected brain is held together by zinc and copper, (ii) that Aβ peptides exhibit Fe/Cu-dependent redox activity similar to that of SOD, (iii) that $A\beta_{1-42}$ is especially redox reactive and has the unusual property of reducing $O_2$ to $O_2^-$, and (iv) that deregulation of Aβ redox reactivity causes the peptide to conveniently polymerize. Since these reactions must be strongly implicated in the pathogenetic events of AD, they offer promising targets for therapeutic drug design.

The discovery that Aβ can generate $H_2O_2$ and Cu(I), both of which are associated with neurotoxic effects, offers an explanation for the neurotoxicity of Aβ polymers. These findings suggest that it may be possible to lessen the neurotoxicity of Aβ by controlling factors which alter the concentrations of Cu(I) and ROS, including hydrogen peroxide, being generated by accumulated and soluble Aβ. It has been discovered that manipulation of factors such as zinc, copper, and pH can result in altered Cu(I) and $H_2O_2$ production by Aβ. Therefore, agents identified as being useful for the adjustment of the pH and

levels of zinc and copper of the brain interstitium can be used to adjust the concentration of Cu(I) and $H_2O_2$, and can therefore be used to reduce the neurotoxic burden. Such agents will thus be a means of treating Alzheimer's disease.

Thus, one object of the present invention is to provide a method for the identification of agents to be used in the treatment of AD. As may be understood by reference to the Examples below, agents to be used in the treatment of AD include:

(a) agents that reduce the amount of Cu(I) or Fe(II) produced by Aβ;

(b) agents that promote or inhibit the production of hydrogen peroxide by Aβ;

(c) agents that inhibit the production of $O_2$- by Aβ;

(d) agents that inhibit the production of OH·.

Of course, as aggregation and especially crosslinking of Aβ contributes to the neurotoxic burden, agents which have been identifed to have the activities listed above may then also be subjected to tests which determine if an agent is capable of inhibiting oligomerization by Aβ (see Example 1).

Agents identified as having the above-listed activities may then be tested for their ability to reduce the neurotoxicity of both soluble and crosslinked Aβ.

Thus, in one aspect, the invention relates to a method for the identification of an agent to be used in the treatment of AD, wherein the agent is capable of altering, and preferably decreasing, the production of Cu(I) by Aβ, the method comprising:

(a) adding Cu(II) to a first Aβ sample;

(b) allowing the first sample to incubate for an amount of time sufficient to allow said first sample to generate Cu(I);

(c) adding Cu(II) to a second Aβ sample, the second sample additionally comprising a candidate pharmacological agent;

(d) allowing the second sample to incubate for the same amount of time as the first sample;

(e) determining the amount of Cu(I) produced by the first sample and the second sample; and

(f) comparing the amount of Cu(I) produced by the first sample to the amount of Cu(I) produced by the second sample;

whereby a difference in the amount of Cu(I) produced by the first sample as compared to the second sample indicates that the candidate pharmacological agent has altered the production of Cu(I) by Aβ. Of course, where the amount of Cu(I) is lower in the second sample than in the first sample, this will indicate that the agent has decreased Cu(I) production.

In a preferred embodiment, the amount of Cu(I) present in said first and said second sample is determined by

(a) adding a complexing agent to said first and said second sample, wherein said complexing agent is capable of combining with Cu(I) to form a complex compound, wherein said complex compound has an optimal visible absorption wavelength;

(b) measuring the absorbancy of said first and said second sample; and

(c) calculating the concentration of Cu(I) in said first and said second sample using the absorbancy obtained in step (b).

In a more preferred embodiment, the complexing agent is bathocuproinedisulfonic (BC) anion. The concentration of $Cu^{+}$ produced by Aβ may then be calculated on the basis of the absorbance of the sample at about 478 nm to about 488 nm, more preferable about 480 to about 486 nm, and most preferably about 483 nm.

In an even more preferred embodiment, the above-described method may be preformed in a microtiter plate, and the absorbancy measurement is performed by a plate reader, thus allowing large numbers of candidate pharmacological compounds to be tested simultaneously.

In another aspect, the invention relates to a method for the identification of an agent to be used in the treatment of AD, wherein said agent is capable of

altering, and preferably decreasing, the production of Fe(II) by Aβ, said method comprising:

(a) adding Fe(III) to a first Aβ sample;

(b) allowing said first sample to incubate for an amount of time sufficient to allow said first sample to generate $Cu^{+}$;

(c) adding Fe(III) to a second Aβ sample, said second sample additionally comprising a candidate pharmacological agent;

(d) allowing said second sample to incubate for the same amount of time as said first sample;

(e) determining the amount of Fe(II) produced by said first sample and said second sample; and

(f) comparing the amount of Fe(II) present in said first sample to the amount of Fe(II) present in said second sample;

whereby a difference in the amount of Fe(II) present in said first sample as compared to said second sample indicates that said candidate pharmacological agent has altered the production of Fe(II) by Aβ. Of course, where the amount of Fe(II) is lower in the second sample than in the first sample, this will indicate that the agent has decreased Fe(II) production.

In a preferred embodiment, the amount of Fe(II) present is determined by using a spectrophotometric method analagous to that used for the determination of Cu(I), above. In this method, the complexing agent is batho-phenanthrolinedisulfonic (BP) anion. The concentration of $Fe^{2+}$-BP produced by Aβ may then be calculated on the basis of the absorbance of the sample at about 530 to about 540 nm, more preferably about 533 nm to about 538 nm, and most preferably about 535 nm.

In an even more preferred embodiment, the above-described method may be preformed in a microtiter plate, and the absorbancy measurement is performed by a plate reader, thus allowing large numbers of candidate pharmacological compounds to be tested simultaneously.

In yet another aspect, the invention relates to a method for the identification of an agent to be used in the treatment of AD, wherein said agent is capable of altering the production of $H_2O_2$ by Aβ, said method comprising:

(a) adding Cu(II) or Fe(III) to a first Aβ sample;

(b) allowing said first sample to incubate for an amount of time sufficient to allow said first sample to generate $H_2O_2$;

(c) adding Cu(II) or Fe(III) to a second Aβ sample, said second sample additionally comprising a candidate pharmacological agent;

(d) allowing said second sample to incubate for the same amount of time as said first sample;

(e) determining the amount of $H_2O_2$ produced by said first sample and said second sample; and

(f) comparing the amount of $H_2O_2$ present in said first sample to the amount of $H_2O_2$ present in said second sample;

whereby a difference in the amount of $H_2O_2$ present in said first sample as compared to said second sample indicates that said candidate pharmacological agent has altered the production of $H_2O_2$ by Aβ. As will be understood by one of ordinary skill in the art, this method may be used to detect agents which decrease the amount of $H_2O_2$ produced (in which case the amount of $H_2O_2$ will be lower in the second sample than in the first sample), or to increase the amount of $H_2O_2$ produced (in which case the amount of $H_2O_2$ will be lower in the first sample than in the second sample).

In a preferred embodiment, the determination of the amount of $H_2O_2$ present in said first and said second sample is determined by

(a) adding catalase to a first aliquot of said first sample obtained in step (a) of claim 1 in an amount sufficient to break down all of the $H_2O_2$ generated by said sample;

(b) adding TCEP, in an amount sufficient to capture all of the $H_2O_2$ generated by said samples, to

(i) said first aliquot

(ii) a second aliquot of said first sample obtained in step (a) of claim 1; and

(iii) said second sample obtained in step (b) of claim 1;

(c) incubating the samples obtained in step (b) for an amount of time sufficient to allow the TCEP to capture all of the $H_20_2$;

(d) adding DTNB to said samples obtained in step (c);

(e) incubating said samples obtained in step (d) for an amount of time sufficient to generate TMB;

(f) measuring the absorbancy at about 407to about 417 nm of said samples obtained in step (e); and

(g) calculating the concentration of $H_2O_2$ in said first and said second sample using the absorbancies obtained in step (f). In a preferred embodiment, the absorbancy of TMB is measured at about 412 nm.

In a preferred embodiment, the above-described method is performed in a microtiter plate, and the absorbancy measurement is performed by a plate reader, thus making it possible to screen large numbers of candidate pharmacological agent simultaneously.

In another enbodiment, the invention provides a method for the identification of an agent to be used in the treatment of AD, wherein said agent is capable of decreasing the production of $O_2$- by Aβ, said method comprising:

(a) adding Aβ and to a first buffer sample having an $O_2$ tension greater than 0;

(b) allowing said first sample to incubate for an amount of time sufficient to allow said first sample to generate $O_2$-;

(c) adding Aβ and a candidate pharmacological agent to a second buffer sample having an $O_2$ tension greater than 0;;

(d) allowing said second sample to incubate for the same amount of time as said first sample;

(e) determining the amount of $O_2$- produced by said first sample and said second sample; and

(f) comparing the amount of $O_2$- present in said first sample to the amount of $O_2$- present in said second sample;

whereby a difference in the amount of $O_2$- present in said first sample as compared to said second sample indicates that said candidate pharmacological agent has altered the production of $O_2$- by Aβ. In a preferred embodiment, the Aβ used is $A\beta_{1-42}$.

Of course, the amount of $O_2$- produced by Aβmay be measured by any method known to those of ordinary skill in the art. In a preferred embodiment, the determination of the amount of $O_2$- present in said samples is accomplished by measuring the absorbancy of the sample at about 250 nm.

Because the ability of Aβ to generate $H_2O_2$ from $O_2$- may in many instances be beneficial, in a prefered embodiment, the invention also relates to a method for the identification of an agent to be used in the treatment of AD, wherein said agent is capable of interfering with the interaction of $O_2$ and Aβ to produce $O_2$-, without interfering with the SOD-like activity of Aβ, said method comprising:

(a) identifying an agent capable of decreasing the production of $O_2$- by Aβ; and

(b) determining the ability of said agent to alter the SOD-like activity of Aβ. In a preferred embodiment, the determination of the ability of said agent to alter the SOD-like activity of Aβ is made by determining whether Aβ is capable of catalytically producing Cu(I), Fe(II) or $H_2O_2$. Methods, besides those which are disclosed elsewhere in this application, for determining if Aβ is capable of catalytically producing Cu(I), Fe(II) or $H_2O_2$ are well known to those of ordinary skill in the art. In particular, the catalytic production of $H_2O_2$ may be determined by using laser flash photolysis or pulse radiolysis (G. Peters and M.A. J. Rodgers, *Biochim. Biophys. Acta 637*: 43-52 (1981)..

In another aspect, candidate pharmacological agents which have been identified by one or more of the above screening assays can undergo further screening to determine if the agents are capable of altering, and preferably

reducing or eliminating, Aβ-mediated toxicity in cell culture. Such assays include the MTT assay, which measures the reduction of 3-(4,5-dimethylthiazol-2-yl)-2,5, diphenyl tetrazolium bromide (MTT) to a colored formazon (Hansen *et al.*, 1989). Although alternatives have not been ruled out (see Burdon *et al.*, 1993), the major site of MTT reduction is thought to be at two stages of electron transport, the cytochrome oxidase and ubiquinone of mitochondria (Slater *et al.*, 1963). A second cytotoxic assay is the release of lactic dehydrogenase (LDH) from cells, a measurement routinely used to quantitate cytotoxicity in cultured CNS cells (Choi, 1987). While MTT measures primarily early redox changes within the cell reflecting the integrity of the electron transport chain, the release of LDH is thought to be through cell lysis. A third assay is visual counting in conjunction with trypan blue exclusion. Other commercially available assays for neurotoxicity, including the Live-Dead assay, may also be used to determine if a candidate compound which alters Cu(I), Fe(II), $H_2O_2$, $OH\cdot$, and $O_2$- production, or alters copper-induced, pH dependent aggregation and crosslinking of Aβ, is also capable of reducing the neurotoxicity of Aβ.

Thus, in another prefered embodiment, the invention relates to a method for the identification of an agent to be used in the treatment of AD, wherein said agent is capable of reducing the toxicity of Aβ, said method comprising:

(a) adding Aβ to a first cell culture;

(b) adding Aβ to a second cell culture, said second cell culture additionally containing a candidate pharmacological agent;

(c) determining the level of neurotoxicity of Aβ in said first and said second samples; and

(d) comparing the level of neurotoxicity of Aβ in said first and said second samples,

whereby a lower neurotoxicity level in said second sample as compared to said first sample indicates that said candidate pharmacological agent has reduced the neurotoxicity of Aβ, and is thereby capable of being used to treat AD.

Assays which can be used to determine the neurotoxicity of a candidate agent include, but are not limited to, the MTT assay and the LDH release assay, as described in Behl *et al.* (*Cell 77*: 817-827 (1994)) and the Live/Dead EukoLight Viability/Cytotoxicity Assay, commercially available from Molecular Probes, Inc. (Eugene, OR).

Cells types which may be used for these neurotoxicity assays include both cancer cells and primary cells, such as rat primary frontal neuronal cells.

Candidate pharmacological agents to be tested in any of the above-described methods will be broad-ranging but can be classified as follows:

Candidate pharmacological agents for the alteration of the SOD-like activity of Aβ will be broad-ranging but can be classified as follows:

*Agents which modify the availability of zinc or copper for interaction with Aβ:* They include chelating agents such as desferrioxamine, but also include amino acids histidine and cysteine which bind free zinc, and are thought to be involved in bringing zinc from the plasma across the blood-brain barrier (BBB). These agents include all classes of specific zinc chelating agents, and combinations of non-specific chelating agents capable of chelating zinc such as EDTA (Edetic acid, N,N′-1,2-Ethane diylbis[N-(carboxymethyl)glycine] or (ethylenedinitrilo)tetraacetic acid, entry 3490 in Merck Index 10th edition) and all salts of EDTA, and/or phytic acid [myo-Inositol hexakis(dihydrogen phosphate), entry 7269 in the Merck Index 10th edition] and phytate salts. Preferred candidate agents within this class include bathocuproine and bathophenanthroline

*Miscellaneous:* Because there is no precedent for an effective anti-amyloidotic pharmaceutical, it is reasonable to serendipitously try out compounds which may have access to the brain compartment for their ability to inhibit either $Cu^{+}$ or $H_2O_2$ production by Aβ. These compounds include dye compounds, heparin, heparan sulfate, and anti-oxidants, *e.g.*, ascorbate, trolox and tocopherols.

In the present invention, the Aβ used may be any form of Aβ. In a preferred embodiment, the Aβ used is selected from the group consisting of $A\beta_{1-39}$, $A\beta_{1-40}$, $A\beta_{1-41}$, $A\beta_{1-42}$, and $A\beta_{1-43}$. Even more preferably, the Aβ used is $A\beta_{1-40}$ or $A\beta_{1-42}$. The most preferred embodiment of the invention makes use of $A\beta_{1-40}$. The sequence of Aβ peptide is found in C. Hilbich *et al., J. Mol. Biol. 228*:460-473 (1992).

The pH of the various reaction mixtures are preferably close to neutral (about 7.4). The pH, therefore, may range from about 6.6 to about 8, preferably from about 6.6 to about 7.8, and most preferably about 7.4.

Buffers which can be used in the methods of the present invention include, but are not limited to, PBS, Tris-chloride and Tris-base, MOPS, HEPES, bicarbonate, Krebs, and Tyrode's. The concentration of the buffers may be between about 10 mM and about 500 mM. Because of the nature of the assays which are included in the methods of the claimed invention, when choosing a buffer, it must be borne in mind that spontaneous free radical production within a given buffer might interfere with the reactions. For this reason, PBS is the preferred buffer for use in the methods of the invention, although other buffers may be used provided that proper controls are used to correct for the above-mentioned free radical formation of a given buffer.

Cu(II) must be present in the reaction mixture for Aβ to produce $Cu^+$. Any salt of Cu(II) may be used to satisfy this requirement, including, but not limited to, $CuCl_2$, $Cu(NO_3)_2$, *etc*. Concentrations of copper from at least about 1 μM may be used; most preferable, a copper concentration of about 10 μM is to be included in the reaction mixture.

Similarly, a redox active metal such as Cu(II) or Fe(III) must be present in the reaction mixture for Aβ to catalytically produce $H_2O_2$. Any salt of Cu(II) may be used to satisfy this requirement, including, but not limited to, $CuCl_2$, $Cu(NO_3)_2$, *etc*. Similarly, and salt of Fe(III) may be used in accordance with the invention, such as $FeCl_3$. Concentrations of copper or iron from at least about 1

μM may be used; most preferably, a copper or iron concentration of about 10 μM is to be included in the reaction mixture.

The present invention may be practiced at temperatures ranging from about 25°C to about 40°C. The preferred temperature range is from about 30°C to about 40°C. The most preferred temperature for the practice of the present invention is about 37°C, *i.e.*, human body temperature.

The production of $Cu^+$ and $H_2O_2$ by Aβ peptide occurs at near-instantaneous rate. Hence, the measurement of the concentration of $Cu^+$ or $H_2O_2$ produced may be performed by the present methods substantially immediately after the addition of Cu(II) to the Aβ peptide. However, if desired, the reaction may be allowed to proceed longer. In a preferred embodiment of the invention, the reaction is carried out for about 30 minutes.

The invention may also be carried out in the presence of biological fluids, such as the preferred biological fluid, CSF, to closely simulate actual physiological conditions. Of course, such fluids will already contain Aβ, so that where the methods of the invention are to be carried out utilizing a biological fluid such as CSF, no further Aβ peptide will be added to the sample. The biological fluid may be used directly or diluted from about 1:1,000 to about 1:5 fold.

The amount of $H_2O_2$ , Cu(I) or Fe(II) produced by a sample may be measured by any standard assay for $H_2O_2$, Cu(I) or Fe(II). For example, the PeroXOquant Qunatitative Peroxide Assay (Pierce, Rockford, IL) may be used to determine the amount of $H_2O_2$ produced. Fe(II) may be determined using the spectrophotometric method of Linert *et al.* (*Biochim. Biophys. Acta 1316*:160-168 (1996)). Other such methods will be readily apparent to those of ordinary skill in the art.

In a preferred embodiment, the $H_2O_2$ or $Cu^+$ produced by the sample is complexed with a complexing agent having an optimal visible absorption wavelength. The amount of $H_2O_2$ or $Cu^+$ produced by a sample is then detected using optical spectrophotometry (see Example 2).

In a preferred embodiment, the complexing agent to be used for the determination of the amount of $Cu^+$ produced is bathocuproinedisulfonic anion (BC), (see Example 2); the complex $Cu^+$-BC has an optimal visible absorption wavelength of about 483 nm. As is mentioned above, Aβ will produce $H_2O_2$ and $Cu^+$almost immediately following the addition of Cu(II) and Zn(II) to the reaction mixture. Thus, BC may be added to the reaction immediately following the addition of Cu(II) and Zn(II) to the Aβ samples. The concentration of BC to be achieved in a sample is between about 10 μM to about 400 μM, more preferably about 75 μM to about 300 μM, and still more preferably about 150 μM to about 275 μM. In the most preferred embodiment, the concentration of BC to be achieved in a sample is about 200 μM. Of course, one of ordinary skill in the art can easily optimize the concentration of BC to be added with no more than routine experimentation.

Where the amount of Fe(II) produced is to be determined, the complexing agent to be used for the determination of the amount of Fe(II) produced is bathophenanthrolinedisulfonic (BP) anion, (see Example 2); the complex $Fe^{2+}$-BP has an optimal visible absorption wavelength of about 535 nm. As is mentioned above, Aβ will produce $H_2O_2$ and Fe(II) almost immediately following the addition of Fe(III) and Zn(II) to the reaction mixture. Thus, BP may be added to the reaction immediately following the addition of Fe(III) and Zn(II) to the Aβ samples. The concentration of BP to be achieved in a sample is between about 10 μM to about 400 μM, more preferably about 75 μM to about 300 μM, and still more preferably about 150 μM to about 275 μM. In the most preferred embodiment, the concentration of BP to be achieved in a sample is about 200 μM. Of course, one of ordinary skill in the art can easily optimize the concentration of BP to be added with no more than routine experimentation.

The above-described spectrophotometric assays may be used to determine the concentration of $Cu^+$ or $Fe^{2+}$, as is described in Example 2.

Each of the assays of the present invention is ideally suited for the preparation of a kit. Such a kit may comprise a carrier means being

compartmentalized to receive in close confinement therein one or more container means, such as vials, tubes, and the like, each of said container means comprising one of the separate elements of the assay to be used in the method. For example, there may be provided a container means containing standard solutions of the Aβ peptide or lyophilized Aβ peptide and a container means containing a standard solution or varying amounts of a salt of redox active metal, such as Cu(II) or Fe(III), in any form, *i.e.*, in solution or dried, soluble or insoluble, in addition to further carrier means containing varying amounts and/or concentrations of reagents used in the present methods. For example, solutions to be used for the determination of Cu(I) or Fe(II) as described in Example 2 will include BC anion and BP anion, respectively. Similarly, solutions to be used for the determination of $H_2O_2$ as described in Example 2 include TCEP and DTNB, as well as catalase (10U/ml). Standard solutions of Aβ peptide preferably have concentrations above about 10 $\mu$M, more preferably from about 10 to about 25 $\mu$M or if the peptide is provided in its lyophilized form, it is provided in an amount which can be solubilized to said concentrations by adding an aqueous buffer or physiological solution. The standard solutions of analytes may be used to prepare control and test reaction mixtures for comparison, according to the methods of the present invention.

The following examples are provided by way of illustration to further describe certain preferred embodiments of the invention, and are not intended to be limiting of the present invention, unless specified.

# *Examples*

## *Example 1*

### *Copper-Induced, pH Dependent Aggregation of Aβ*

***Materials and Methods***

***a) Preparation of Aβ Stock***

Human $Aß_{1-40}$ peptide was synthesized, purified and characterized by HPLC analysis, amino acid analysis and mass spectroscopy by W.M. Keck Foundation Biotechnology Resource Laboratory (Yale University, New Haven, CT). Synthetic Aß peptide solutions were dissolved in trifluoroethanol (30 % in Milli-Q water (Millipore Corporation, Milford, MA)) or 20 mM Hepes (pH 8.5) at a concentration of 0.5-1.0 g/ml, centrifuged for 20 min. at 10 000g and the supernatant (stock $Aß_{1-40}$) used for subsequent aggregation assays on the day of the experiment. The concentration of stock $Aß_{1-40}$ was determined by UV spectroscopy at 214 nm or by Micro BCA protein assay (Pierce, Rockford, IL). The Micro BCA assay was performed by adding 10µl of stock $Aß_{1-40}$ (or bovine serum albumin standard) to 140 µl of distilled water, and then adding an equal volume of supernatant (150µl) to a 96-well plate and measuring the absorbance at 562 nm. The concentration of $Aß_{1-40}$ was determined from the BSA standard curve. Prior to use all buffers and stock solutions of metal ions were filtered though a 0.22 µm filter (Gelan Sciences, Ann Arbor, MI) to remove any particulate matter. All metal ions were the chloride salt, except lead nitrate.

***b) Aggregation Assays***

$Aß_{1-40}$ stock was diluted to 2.5 µM in 150 mM NaCl and 20 mM glycine (pH 3-4.5), mes (pH 5-6.2) or Hepes (pH 6.4-8.8), with or without metal ions,

incubated (30 min., 37 °C), centrifuged (20 min., 10 000g). The amount of protein in the supernatant was determined by the Micro BCA protein assay as described above.

### *c) Turbidometric Assays*

Turbidity measurements were performed as described by Huang *et al.*, *Journal of Biological Chemistry* (submitted), except $A\beta_{1-40}$ stock was brought to 10 μM (300 μl) in 20 mM HEPES buffer, 150 mM NaCl (pH 6.6, 6.8 or 7.4) with or without metal ions prior to incubation (30 min., 37 °C). To investigate the pH reversibility of $Cu^{2+}$-induced Aß aggregation, 25 μM $A\beta_{1-40}$ and 25 μM $Cu^{2+}$ were mixed in 67 mM phosphate buffer, 150 mM NaCl (pH 7.4) and turbidity measurements were taken at four 1 min. intervals. Subsequently, 20 μl aliquots of 10 mM EDTA or 10 mM $Cu^{2+}$ were added into the wells alternatively, and, following a 2 min. delay, a further four readings were taken at 1 min. intervals. After the final EDTA addition and turbidity reading, the mixtures were incubated for an additional 30 min. before taking final readings. To investigate the reversibility of pH mediated $Cu^{2+}$-induced $A\beta_{1-40}$ aggregation, 10 μM $A\beta_{1-40}$ and 30 μM $Cu^{2+}$ were mixed in 67 mM phosphate buffer, 150 mM NaCl (pH 7.4) and an initial turbidity measurement taken. Subsequently, the pH of the solution was successively decreased to 6.6 and then increased back to 7.5. The pH of the reaction was monitored with a microprobe (Lazar Research Laboratories Inc., Los Angeles, CA) and the turbidity read at 5 min. intervals for up to 30 min. This cycle was repeated three times.

### *d) Immunofiltration Detection of Low Concentrations of $A\beta_{1-40}$ Aggregate*

Physiological concentrations of $A\beta_{1-40}$ (8 nM) were brought to 150 mM NaCl, 20 mM HEPES (pH 6.6 or 7.4), 100 nM BSA with $CuCl_2$ (0, 0.1, 0.2, 0.5 and 2 μM) and incubated (30 min., 37°C). The reaction mixtures (200 μl) were

then placed into the 96-well Easy-Titer ELIFA system (Pierce, Rockford, IL) and filtered through a 0.22 μm cellulose acetate filter (MSI, Westboro, MA). Aggregated particles were fixed to the membrane (0.1% glutaraldehyde, 15 min.), washed thoroughly and then probed with the anti-Aß mAB 6E10 (Senetek, Maryland Heights, MI). Blots were washed and exposed to film in the presence of ECL chemiluminescence reagents (Amersham, Buckinghamshire, England). Immunoreactivity was quantified by transmitance analysis of ECL film from the immunoblots.

***e) Aß metal-capture ELISA***

$Aß_{1-40}$ (1.5 ng/well) was incubated (37°C, 2 hr) in the wells of $Cu^{2+}$ coated microtiter plates (Xenopore, Hawthorne, NJ) with increasing concentrations of $Cu^{2+}$ (1-100 nM) as described by Moir *et al.*, *Journal of Biological Chemistry* (submitted). Remaining ligand binding sites on well surfaces were blocked with 2% gelatin in tris-buffered saline (TBS) (3 hr at 37°C) prior to overnight incubation at room temperature with the anti-Aß mAb 6E10 (Senetek, Maryland Heights, MI). Anti-mouse IgG coupled to horseradish peroxidase was then added to each well and incubated for 3 hr at 37°C. Bound antibodies were detected by a 30 minute incubation with stable peroxidase substrate buffer/3,3',5,5'-Tetramethyl benzidine (SPSB/TMB) buffer, followed by the addition of 2 M sulfuric acid and measurement of the increase in absorbance at 450 nm.

***f) Extraction of Aβ from post-mortem brain tissue***

Identical regions of frontal cortex (0.5g) from post-mortem brains of individuals with AD, as well as non-AD conditions, were homogenized in TBS, pH 4.7 ±metal chelators. The homogenate was centrifuged and samples of the soluble supernatant as well as the pellet were extracted into SDS sample buffer and assayed for Aβ content by western blotting using monoclonal antibody

(mAb) WO2. The data shows a typical (of n=12 comparisons) result comparing the amount of Aβ extracted into the supernatant phase in AD compared to control (young adult) samples. N,N,N',N'-tetrakis [2-pyridyl-methyl] ethylenediamine (TPEN) (5 μM) allows the visualization of a population of pelletable Aβ that had not previously been recognized in unaffected brain samples (Fig. 8).

***g) Aβ cross-linking by copper***

$Cu^{2+}$-induced SDS-resistant oligomerization of Aβ: $Aβ_{1-40}$ (2.5 μM), 150 mM NaCl, 20 mM hepes (pH 6.6, 7.4, 9) with or without $ZnCl_2$ or $CuCl_2$. Following incubation (37°C), aliquots of each reaction (2 ng peptide) were collected at 0 d, 1 d, 3 d and 5 d and western blotted using anti-Aβ monoclonal antibody 8E10. Migration of the molecular size markers are indicated (kDa). The dimer formed under these conditions has been found to be covalently linked. Cu(II) (2-30 μM) induced and covalent oligomerization of peptide. Co-incubation with similar concentrations of Zn(II) accelerates the bridging, but zinc alone has no effect. The antioxidant sodium metabisulphite moderately attenuates the reaction, while ascorbic acid dramatically accelerates Aβ bridging. This suggests reduction of Cu(II) to Cu(I) with the latter mediating covalent bridging of Aβ. Mannitol also abolishes the cross-linking, suggesting that the bridging is mediated by the generation of the hydroxyl radical by a Fenton reaction that recruits Cu(I) (Fig. 9). It should be noted that other means of visualizing and/or determining the presence or absence of crosslinking other than western blot analysis may be used. Such other means include but are not limited to density sedimentation by centrifugation of the samples.

***Results***

It has previously been reported that $Zn^{2+}$ induces rapid precipitation of Aβ *in vitro* (Bush, A.I., *et al.*, *Science 268*:1921 (1995)). This metal has an abnormal metabolism in AD and is highly concentrated in brain regions where Aβ precipitates. The present data indicate that under very slightly acidic conditions, such as in the lactic acidotic AD brain, $Cu^{2+}$ strikingly induces the precipitation of Aβ through an unknown conformational shift. pH alone dramatically affects Aβ solubility, inducing precipitation when the pH of the incubation approaches the pl of the peptide (pH 5-6). Zinc induces 40-50% of the peptide to precipitate at pH > 6.2, below pH 6.2 the precipitating effects of $Zn^{2+}$ and acid are not summative. At pH ≤5, $Zn^{2+}$ has little effect upon Aβ solubility. $Cu^{2+}$ is more effective than $Zn^{2+}$ in precipitating Aβ and even induces precipitation at the physiologically relevant pH 6-7. Copper-induced precipitation of Aβ occurs as the pH falls below 7.0, comparable with conditions of acidosis (Yates, C.M., *et al.*, *J. Neurochem. 55*:1624 (1990)) in the AD brain. Investigation of the precipitating effects of a host or other metal ions in this system indicated that metal ion precipitation of Aβ was limited to copper and zinc, as illustrated, although Fe(II) possesses a partial capacity to induce precipitation (Bush, A.I., *et al.*, *Science 268*:1921 (1995)).

On the basis these *in vitro* findings, the possibility that Aβ deposits in the Ad-affected brain may be held in assembly by zinc and copper ions was investigated. Roher and colleagues have recently shown that much of the Aβ that deposits in Ad-affected cortex can be solubilized in water (Roher, A.E, *et al.*, *J. Biol. Chem. 271*:20631 (1996)). Supporting the clinical relevance of *in vitro* findings, it has recently been demonstrated that metal chelators increase the amount of Aβ extracted by Roher's technique (in neutral saline buffer), and that the extraction of Aβ is increased as the chelator employed has a higher affinity for zinc or copper (FIG. 8). Hence TPEN is highly efficient in extracting Aβ, as are TETA, and bathocuproene, EGTA and EDTA are less efficient, requiring

higher concentrations 91 mm) to achieve the same level of recovery as say, TPEN (5μM). Zinc and copper ions (5-50 μM) added back to the extracting solution abolish the recovery of Aμ (which is subsequently extracted by the SDS sample buffer in the pellet fraction of the centrifuged brain homogenate suspension), but $Ca^{2+}$ and $Mg^{2+}$ added back to the chelator-mediated extracts of Aβ cannot abolish Aβ resolubilization from Ad-affected tissue even when these metal ions are present in millimolar concentrations.

Importantly, atomic absorption spectrophotometry assays of the metal content of the chelator-mediated extracts confirms that Cu and Zn are co-released with Aβ by the chelators, along with lower concentrations of Fe. These data strongly indicate that Aβ deposits (probably of the amorphous type) are held together by Cu and Zn and may also contain Fe. Interestingly, Aβ is not extractable from control brain without the use of chelators. This suggests that metal-assembled Aβ deposits may be the earliest step in the evolution of Aβ plaque pathology.

These findings propelled further inquiries into chemistry of metal ion- Aβ interaction. The precipitating effects upon Aβ of Zn(II) and Cu(II) were found to be qualitatively different. Zn-mediated aggregation is reversible with chelation and is not associated with neurotoxicity in primary neuronal cell cultures, whereas Cu-mediated aggregation is accompanied by the slow formation of covalently-bonded SDS-resistant dimers and induction of neurotoxicity. These neurotoxic SDS-resistant dimers are similar to those described by Roher (Roher, A.E, *et al.*, *J. Biol. Chem. 271*:20631 (1996)).

To accurately quantitate the effects of different metals and pH on Aß solubility, synthetic human $Aß_{1-40}$ (2.5 μM) was incubated (37°C) in the presence of metal ions at various pH for 30 min. The resultant aggregated particles were sedimented by centrifugation to permit determination of soluble $Aß_{1-40}$ in the supernatant. To determine the centrifugation time required to completely sediment the aggregated particles generated under these conditions, $Aß_{1-40}$ was incubated for 30 min at 37°C with no metal, $Zn^{2+}$ (100μM), $Cu^{2+}$ (100 μM) and

pH (5.5). Reaction mixtures were centrifuged at 10 000g for different times, or ultracentrifuged at 100 000g for 1 h. (FIG. 1). Figure 1 shows the proportion of soluble $A\beta_{1-40}$ remaining following centrifugation of reaction mixtures. $A\beta_{1-40}$ was incubated (30 min., 37 °C) with no metal, under acidic conditions (pH 5.5), $Zn^{2+}$ (100 μM) or $Cu^{2+}$ (100 μM), and centrifuged at 10 000*g* for different time intervals, or at 100,000*g* (ultracentrifuged) for 1 h for comparison. All data points are means ± SD, n = 3.

Given that conformational changes within the N-terminal domain of Aß are induced by modulating $[H^+]$ (Soto, C., *et al., J. Neurochem. 63*:1191-1198 (1994)), and that there is a metal ($Zn^{2+}$) binding domain in the same region, experiments were designed to determine whether there was a synergistic effect of pH on metal ion-induced Aß aggregation. $A\beta_{1-40}$ was incubated with different bioessential metal ions at pH 6.6, 6.8 and 7.4. The results are show in FIG. 2A, where "all metals" indicates incubation with a combination containing each metal ion at the nominated concentrations, concurrently. FIG. 2A shows the proportion of soluble $A\beta_{1-40}$ remaining in the supernatant after incubation (30 min., 37°C) with various metals ions at pH 6.6, 6.8 or 7.4 after centrifugation (10,000g, 20 min.),

The $[H^+]$ chosen represented the most extreme, yet physiologically plausible $[H^+]$ that $A\beta_{1-40}$ would be likely to encounter *in vivo*. The ability of different bioessential metal ions to aggregate $A\beta_{1-40}$ at increasing $H^+$ concentrations fell into two groups; $Mg^{2+}$, $Ca^{2+}$, $Al^{3+}$, $Co^{2+}$, $Hg^{2+}$, $Fe^{3+}$, $Pb^{2+}$ and $Cu^{2+}$ showed increasing sensitivity to induce $A\beta_{1-40}$ aggregation, while $Fe^{2+}$, $Mn^{2+}$, $Ni^{2+}$, and $Zn^{2+}$ were insensitive to alterations in $[H^+]$ in their ability to aggregate $A\beta_{1-40}$. $Cu^{2+}$ and $Hg^{2+}$ induced most aggregation as the $[H^+]$ increased, although the $[H^+]$ insensitive $Zn^{2+}$-induced aggregation produced a similar amount of aggregation. $Fe^{2+}$, but not $Fe^{3+}$, also induced considerable aggregation as the $[H^+]$ increased, possibly reflecting increased aggregation as a result of increased crosslinking of the peptide.

Similar results were obtained when these experiments were repeated using turbidometry as an index of aggregation (FIG. 2B). The data indicate the absorbance changes between reaction mixtures with and without metal ions at pH 6.6, 6.8 or 7.4. Thus, $A\beta_{1-40}$ has both a pH insensitive and a pH sensitive metal binding site. At higher concentrations of metal ions this pattern was repeated, except $Co^{2+}$ and $Al^{3+}$-induced Aß aggregation became pH insensitive, and Mn became sensitive (FIG. 2C).

Since $^{64}Cu$ is impractically short-lived (t1/2 = 13 h), a novel metal-capture ELISA assay was used to perform competition analysis of $A\beta_{1-40}$ binding to a microtiter plate impregnated with $Cu^{2+}$, as described in Materials and Methods. Results are shown in FIG. 3. All assays were performed in triplicate and are means ± SD, n=3. Competition analysis revealed that $A\beta_{1-40}$ has at least one high affinity, saturable $Cu^{2+}$ binding site with a Kd = 900 pM at pH 7.4 (FIG. 3). The affinity of Aß for $Cu^{2+}$ is higher than that for $Zn^{2+}$ (Bush, A.I., *et al., J. Biol. Chem. 269*:12152 (1994)). Since $Cu^{2+}$ does not decrease $Zn^{2+}$-induced aggregation (Bush, A.I., *et al., J. Biol. Chem. 269*:12152 (1994)), indicating $Cu^{2+}$ does not displace bound $Zn^{2+}$, there are likely to be two separate metal binding sites. This is supported by the fact that there is both a pH sensitive and insensitive interaction with different metal ions.

Since the conformational state and solubility of Aß is altered at different pH (Soto, C., *et al., J. Neurochem. 63*:1191-1198 (1994)), the effects of $[H^+]$ on $Zn^{2+}$- and $Cu^{+}$-induced $A\beta_{1-40}$ aggregation were studied. Results are shown in FIGS. 4A, 4B and 4C. FIG. 4A shows the proportion of soluble $A\beta_{1-40}$ remaining in the supernatant following incubation (30 min., 37°C) at pH 3.0-8.8 in buffered saline ± $Zn^{2+}$ (30 μM) or $Cu^{2+}$ (30 μM) and centrifugation (10 000g, 20 min.), expressed as a percentage of starting peptide. All data points are means ± SD, n=3. $[H^+]$ alone precipitates $A\beta_{1-40}$ (2.5 μM) as the solution is lowered below pH 7.4, and dramatically once the pH falls below 6.3 (FIG. 4A). At pH 5.0, 80% of the peptide is precipitated, but the peptide is not aggregated by acidic environments below pH 5, confirming and extending earlier reports on the effect

of pH on Aß solubility (Burdick, D., *J. Biol. Chem.* 267:546-554 (1992)). $Zn^{2+}$ (30 μM) induced a constant level (~50%) of aggregation between pH 6.2-8.5, while below pH 6.0, aggregation could be explained solely by the effect of $[H^+]$.

In the presence of $Cu^{2+}$ (30 μM), a decrease in pH from 8.8 to 7.4 induced a marked drop in $Aß_{1-40}$ solubility, while a slight decrease below pH 7.4 strikingly potentiated the effect of $Cu^{2+}$ on the peptide's aggregation. Surprisingly, $Cu^{2+}$caused >85 % of the available peptide to aggregate by pH 6.8, a pH which plausibly represents a mildly acidotic environment. Thus, conformational changes in Aß brought about by small increases in $[H^+]$ result in the unmasking of a second metal binding site that leads to its rapid self-aggregation. Below pH 5.0, the ability of both $Zn^{2+}$ and $Cu^{2+}$ to aggregate Aß was diminished, consistent with the fact that Zn binding to Aß is abolished below pH 6.0 (Bush, A.I., *et al., J. Biol. Chem. 269*:12152 (1994)), probably due to protonation of histidine residues.

The relationship between pH and $Cu^{2+}$ on $Aß_{1-40}$ solubility was then further defined by the following experiments (FIG. 4B). The proportion of soluble $Aß_{1-40}$ remaining in the supernatant after incubation (30 min., 37 °C) at pH 5.4-7.8 with different $Cu^{2+}$ concentrations (0, 5, 10, 20, 30 μM), and centrifugation (10,000g, 20 min.), was measured andexpressed as a percentage of starting peptide. All data points are means ± SD, n=3. At pH 7.4, $Cu^{2+}$-induced Aß aggregation was 50% less than that induced by $Zn^{2+}$over the same concentration range, consistent withearlier reports (Bush, A.I., *et al., J. Biol. Chem. 269*:12152 (1994)). There was a potentiating relationship between $[H^+]$ and $[Cu^{2+}]$ in producing Aß aggregation; as the pH fell, less $Cu^{2+}$was required to induce the same level of aggregation, suggesting that $[H^+]$ is controlling $Cu^{2+}$ induced $Aß_{1-40}$ aggregation.

To confirm that this reaction occurs at physiological concentrations of $Aß_{1-40}$ and $Cu^{2+}$, a novel filtration immunodetection system was employed. This technique enabled the determination of the relative amount of $Aß_{1-40}$ aggregation in the presence of different concentrations of $H^+$ and $Cu^{2+}$ (FIG. 4C). The relative aggregation of nM concentrations of $Aß_{1-40}$ at pH 7.4 and pH 6.6 in the presence

of different $Cu^{2+}$ concentrations (0, 0.1, 0.2, 0.5 μM) were determined by this method. Data represent mean reflectance values of immunoblot densitometry expressed as a ratio of the signal obtained when the peptide is treated in the absence of $Cu^{2+}$. All data points are means ± SD, n = 2.

This sensitive technique confirmed that physiological concentrations of $Aß_{1-40}$ are aggregated under mildly acidic conditions and that aggregation was greatly enhanced by the presence of $Cu^{2+}$ at concentrations as low as 200 nM. Furthermore, as previously observed at higher $Aß_{1-40}$ concentrations, a decrease in pH from 7.4 to 6.6 potentiated the effect of $Cu^{2+}$ on aggregation of physiological concentrations of $Aß_{1-40}$. Thus, $Aß_{1-40}$ aggregation is concentration independent down to 8 nM where $Cu^{2+}$ is available.

It has recently been shown that $Zn^{2+}$ mediated $Aß_{1-40}$ aggregation is reversible whereas $Aß_{1-40}$ aggregation induced by pH 5.5 was irreversible (**57**). Therefore, we testedexperiments were performed to determine whether $Cu^{2+}$/pH-mediated $Aß_{1-40}$ aggregation was reversible. $Cu^{2+}$-induced $Aß_{1-40}$ aggregation at pH 7.4 was reversible following EDTA chelation, although for each new aggregation cycle, complete resolubilization of the aggregates required a longer incubation. This result suggested that a more complex aggregate is formed during each subsequent aggregation cycle, preventing the chelator access to remove $Cu^{2+}$ from the peptide. This is supported by the fact that complete resolubilization occurs with time, and indicates that the peptide is not adopting a structural conformation that is insensitive to $Cu^{2+}$-induced aggregation/EDTA-resolubilization.

The reversibility of pH potentiated $Cu^{2+}$-induced $Aß_{1-40}$ aggregation was studied by turbidometry between pH 7.5 to 6.6, representing $H^+$ concentration extremes that might be found *in vivo* (FIGS. 5A and 5B). Unlike the irreversible aggregation of $Aß_{1-40}$ observed at pH 5.5. $Cu^{2+}$-induced $Aß_{1-40}$ aggregation was fully reversible as the pH oscillated between pH 7.4 and 6.6. Figure 5A shows the turbidometric analysis of $Cu^{2+}$-induced $Aß_{1-40}$ aggregation at pH 7.4 reversed by successive cycles of chelator (EDTA), as indicated. Figure 5B shows

turbidometric analysis of the reversibility of $Cu^{2+}$-induced $A\beta_{1-40}$ as the pH cycles between 7.4 and 6.6. Thus, subtle conformational changes within the peptide induced by changing $[H^+]$ within a narrow pH window, that corresponds to physiologically plausible $[H^+]$, allows the aggregation or resolubilization of the peptide in the presence of $Cu^{2+}$.

***Discussion***

These results suggest that subtle conformational changes in Aß induced by $[H^+]$ promote the interaction of $A\beta_{1-40}$ with metal ions, in particular $Cu^{2+}$and $Hg^{2+}$ allowing self-aggregate or resolubilize depending on the $[H^+]$ (FIGs. 2A, 2B, 2C, 4A, 4B, 4C). A decrease in pH below 7.0 increases the ß-sheet conformation (Soto, C., *et al., J. Neurochem. 63*:1191-1198 (1994)), and this may allow the binding of $Cu^{2+}$ to soluble Aß that could further alter the conformation of the peptide allowing for self aggregation, or simply help coordinate adjacent Aß molecules in the assembly of the peptides into aggregates. Conversely, increasing pH above 7.0 promotes the a-helical conformation (Soto, C., *et al., J. Neurochem. 63*:1191-1198 (1994)), which may alter the conformational state of the dimeric aggregated peptide, releasing Cu and thereby destabilizing the aggregate with the resultant release of Aß into solution. Thus, in the presence of $Cu^{2+}$, $A\beta_{1-40}$ oscillates between an aggregated and soluble state dependent upon the $[H^+]$.

Interestingly, $Hg^{2+}$ mediated Aß aggregation has greatly potentiated by mild acidity (FIG. 2C). $A\beta_{1-40}$ aggregation by $Co^{2+}$, like $Zn^{2+}$, was pH insensitive and per mole induced a similar level of aggregation. Unlike $Zn^{2+}$, $A\beta_{1-40}$ binding of $Co^{2+}$ may be employed for the structural determination of the pH insensitive binding site given its nuclear magnetic cababilities.

The biphasic relationship of Aß solubility with pH mirrors the conformational changes previously observed by CD spectra within the N-terminal fragment (residues 1-28) of Aß (reviewed in (Soto, C., *et al., J. Neurochem.*

*63*:1191-1198 (1994)); a-helical between pH 1-4 and >7, but ß-sheet between pH 4-7. The irreversible aggregates of Aß formed at pH 5.5 supports the hypothesis that the ß-sheet conformation is a pathway for Aß aggregation into amyloid fibrils. Since aggregates produced by $Zn^{2+}$ and $Cu^{2+}$ under mildly acidic conditions (FIGs. 5A and 5B) are chelator/pH reversible, there conformation may be the higher energy a-helical conformation.

These results now indicate that there are three physiologically plausible conditions which could aggregate Aß: pH (FIGs. 1, 4A-4C; Fraser, P.E., *et al.*, *Biophys. J. 60*:1190-1201 (1991); Barrow, C.J. and Zagorski, M.G., *Science 253*:179-182 (1991); Burdick, D., *J. Biol. Chem.* 267:546-554 (1992); Barrow, C.J., *et al., J. Mol. Biol. 225*:1075-1093 (1992); Zagorski, M.G. and Barrow, C.J., *Biochemistry 31*:5621-5631 (1992); Kirshenbaum, K. and Daggett, V., *Biochemistry 34*:7629-7639 (1995); Wood, S.J., *et al., J. Mol. Biol. 256*:870-877 (1996), $[Zn^{2+}]$ (FIGs. 1, 2A and 2B, 4A-4C; Bush, A.I., *et al., J. Biol. Chem. 269*:12152 (1994); Bush, A.I., *et al., Science 265*:1464 (1994); Bush, A.I., *et al., Science 268*:1921 (1995); Wood, S.J., *et al., J. Mol. Biol. 256*:870-877 (1996))and under mildly acidic conditions, $[Cu^{2+}]$ (FIGs. 2A, 4A-4C, 5B). Interestingly, changes in metal ion concentrations and pH are common features of the inflammatory response to injury. Therefore, the binding of $Cu^{2+}$ and $Zn^{2+}$ to Aß may be of particular importance during inflammatory processes, since local sites of inflammation can become acidic (Trehauf, P.S. and McCarty, D.J., *Arthr. Rheum. 14*:475-484 (1971); Menkin, V., *Am. J. Pathol. 10*:193-210 (1934)) and both $Zn^{2+}$ and $Cu^{2+}$ are rapidly mobilized in response to inflammation (Lindeman, R.D., *et al., J. Lab. Clin. Med. 81*:194-204 (1973); Terhune, M.W. and Sandstead, H.H., *Science 177*:68-69 (1972); Hsu, J.M., *et al., J. Nutrition 99*:425-432 (1969); Haley, J.V., *J. Surg. Res. 27*:168-174 (1979); Milaninio, R., *et al., Advances in Inflammation Research 1*:281-291 (1979); Frieden, E., in *Inflammatory Diseases and Copper*, Sorenson, J.R.J., ed, Humana Press, New Jersey (1980), pp. 159-169).

Serum copper levels increase during inflammation, associated with increases in ceruloplasmin, a $Cu^{2+}$ transporting protein that may donate $Cu^{2+}$ to enzymes active in processes of basic metabolism and wound healing such as cytochrome oxidase and lysyl oxidase (Giampaolo, V., *et al.*, in *Inflammatory Diseases and Copper*, Sorenson, J.R.J., ed, Humana Press, New Jersey (1980), pp. 329-345; Peacock, E.E. and vanWinkle, W., in *Wound Repair*, W.B. Saunders Co., Philadelphia (1976), pp. 145-155)). Since the release of $Cu^{2+}$ from ceruloplasmin is greatly facilitated by acidic environments where the cupric ion is reduced to its cuprous form (Owen, C.A., Jr., *Proc. Soc. Exp. Biol. Med. 149*:681-682 (1975)), periods of mild acidosis may promote an environment of increased free $Cu^{2+}$. Similarily, aggregation of another amyloid protein, the acute phase reactant serum amyloid P component (SAP) to the cell wall polysaccharide, zymosan, has been observed with $Cu^{2+}$ at acidic pH (Potempa, L.A., *et al., Journal of Biological Chemistry 260*:12142-12147 (1985)). Thus, exchange of $Cu^{2+}$ to $A\beta_{1-40}$ during times of decreased pH may provide a mechanism for altering the biochemical reactivity of the protein required by the cell under mildly acidic conditions. Such a function may involve alterations in the aggregation/adhesive properties (FIGS. 1-5B) or oxidative functions of Aß at local sites of inflammation.

While the pathogenic nature of $A\beta_{1-42}$ in AD is well described (Maury, C.P.J., *Lab. Investig. 72*:4-16 (1995); Multhaup, G., *et al., Nature 325*:733-736 (1987)), the function of the smaller $A\beta_{1-40}$ remains unclear. The present data suggest that $Cu^{2+}$-binding and aggregation of Aß will occur when the pH of the microenvironment rises. This conclusion can be based on the finding that the reaction is $[H^+]$ and $[Cu^{2+}]$ dependent and reversible within a narrow, physiologically plausible, pH window. This is further supported by the specificity and high affinity of $Cu^{2+}$ binding under mildly acidic conditions compared to the constant $Zn^{2+}$-induced aggregation (and binding) of $A\beta_{1-40}$ over a wide pH range (6.2-8.5). The brain contains high levels of both $Zn^{2+}$ (~150 μM; Frederickson, C.J. *International Review of Neurobiology* 31:145-237 (1989)) and $Cu^{2+}$ (~100

μM; Warren, P.J., *et al., Brain 83*:709-717 (1960); Owen, C.A., *Physiological Aspects of Copper*, Noyes Publications, Park Ridge, New Jersey (1982), pp160-191). Intracellular concentrations are approximately 1000 and 100 fold higher than extracellular concentrations. This large gradient between intracellular and extracellular compartments suggests a highly energy dependent mechanism is required in order to sequester these metals within neurons. Therefore, any alterations in energy metabolism, or injury, may affect the reuptake of these metal ions and promote there release into the extracellular space, and together with the synergistic affects of decreased pH (see above) induce membrane bound $A\beta_{1-40}$ to aggregate. Since increased concentrations of $Zn^{2+}$ and $Cu^{2+}$, and decreased pH, are common features of all forms of cellular insult, the initiation of $A\beta_{1-40}$ function likely occurs in a coordinated fashion to alter adhesive and/or oxidative properties of this membrane protein essential for maintaining cell integrity and viability. That $A\beta_{1-40}$ has such a high affinity for these metal ions, indicates a protein that has evolved to respond to slight changes in the concentration of extracellular metal ions. This is supported by the fact that aggregation in the presence of Cu is approx. 30% at pH 7.1, the pH of the brain (Yates CM, *et al., J. Neurochem. 55*:1624-1630 (1990)), but 85% at pH 6.8. Taken together, our present results indicate that $A\beta_{1-40}$ may have evolved to respond to biochemical changes associated with neuronal damage as part of the locally mediated response to inflammation or cell injury. Thus, it is possible that $Cu^{2+}$ mediated $A\beta_{1-40}$ binding and aggregation might be a purposive cellular response to an environment of mild acidosis.

The deposition of amyloid systemically is usually associated with an inflammatory response (Pepys, M.B. and Baltz, M.L., *Adv. Immunol. 34*:141-212 (1983); Cohen, A.S., in *Arthritis and Allied Conditions*, D.J. McCarty, ed., Lea and Febiger, Philadelphia (1989), pp. 1273-1293; Kisilevsky, R., *Lab. Investig. 49*:381-390 (1983)). For example, serum amyloid A, one of the major acute phase reactant proteins that is elevated during inflammation, is the precursor of amyloid A protein that is deposited in various tissues during chronic

inflammation, leading to secondary amyloidosis (Gorevic, P.D., *et al., Ann. NY Acad. Sci.*:380-393 (1982)). An involvement of inflammatory mechanisms has been suggested as contributing to plaque formation in AD (Kisilevsky, R., *Mol. Neurobiol. 49*:65-66 (1994)). Acute-phase proteins such as alpha 1-antichymotrypsin and c-reactive protein, elements of the complement system and activated microglial and astroglial cells are consistently found in AD brains.

The rapid appearance, within days of Aß deposits and APP immunoreactivity following head injury (Roberts, G. W., *et al., Lancet. 338*:1422-1423 (1991); Pierce, J.E.S., *et al., Journal of Neuroscience 16*:1083-1090 (1996)), rather than the more gradual accumulation of Aß into more dense core amyloid plaques over months or years in AD may be compatible with the release of $Zn^{2+}$, $Cu^{2+}$ and mild acidosis in this time frame. Thus, pH/metal ion mediated aggregation may form the basis for the amorphous Aß deposits observed in the aging brain and following head injury, allowing the maintenance of endothelial and neuronal integrity while limiting the oxidative stress associated with injury that may lead to a diminishment of structural function.

Since decreased cerebral pH is a complication of aging (Yates CM, *et al., J. Neurochem. 55*:1624-1630 (1990)), these data indicate that Cu and Zn mediated Aß aggregation may be a normal cellular response to an environment of mild acidosis. However, prolonged exposure of Aß to an environment of lowered cerebral pH may promote increased concentrations of free metal ions and reactive oxygen species, and the inappropriate interaction of $Aß_{1-42}$ over time promoting the formation of irreversible Aß oligomers and it's subsequent deposition as amyloid in AD. The reversibility of this pH mediated $Cu^{2+}$ aggregation does however present the potential for therapeutic intervention. Thus, cerebral alkalinization may be explored as a therapeutic modality for the reversibility of amyloid deposition *in vivo*.

## *Example 2*

### *Free Radical Formation and SOD-like activity of Alzheimer's Aβ Peptides*

#### *a) Determination of $Cu^{+}$ and $Fe^{2+}$*

This method is modified from a protocol assaying serum copper and iron (Landers, J.W. and Zak, B., *Chim. Acta. 29*:590 (1958)). It is based on the fact that there are optimal visible absorption wavelengths of 483 nm and 535 nm for $Cu^{+}$ complexed with bathocuproinedisulfonic (BC) anion and $Fe^{2+}$ coordinated by bathophenanthrolinedisulfonic (BP) anion, respectively.

Determination of molar absorption of these two complexes was accomplished essentially as follows. An aliquot of 500 µl of each complex (500 µM, in PBS pH 7.4, with ligands in excess) was pipetted into 1 cm-pathlength quartz cuvette, and their absorbances were measured. Their molar absorbancy was determined based on Beer-Lambert's Law. $Cu^{+}$-BC has a molar absorbancy of 2762 $M^{-1}$ $cm^{-1}$, while $Fe^{2+}$-BP has a molar absorbancy of 7124 $M^{-1}$ $cm^{-1}$.

Determination of the equivalent vertical pathlength for $Cu^{+}$-BC and $Fe^{2+}$-BP in a 96-well plate was carried out essentially as follows. Absorbances of the two complexes with a 500 µM, 100 µM, 50 µM, and 10 µM concentration of relevant metal ions ($Cu^{+}$, $Fe^{2+}$) were determined both by 96-well plate readers (300 µL) and UV-vis spectrometer (500 µL), with PBS, pH 7.4, as the control blank. The resulting absorbancies from the plate reader regress against absorbancies by a UV-vis spectrometer. The slope k from the linear regression line is equivalent to the vertical pathlength if the measurement is carried out on a plate. The results are:

| | k(cm) | $r^2$ |
|---|---|---|
| $Cu^{+}$-BC | 1.049 | 0.998 |
| $Fe^{2+}$-BP | 0.856 | 0.999 |

With molar absorbancy and equivalent vertical pathlength in hand, the concentrations (μM) of $Cu^+$ or $Fe^{2+}$ can be deduced based on Beer-Lambert's Law, using proper buffers as controls.

$$for\ Cu^{+}:\quad [Cu^{+}]\ (\mu M) = \frac{\Delta A(483nm)}{(2762\ x\ 1.049)}\ x\ 10^{6}$$

$$for\ Fe^{2+}:\quad [Fe^{2+}]\ (\mu M) = \frac{\Delta A\ (535nm)}{(7124\ x\ 0.856)}\ x\ 10^{6}$$

where ΔA is absorbancy difference between sample and control blank.

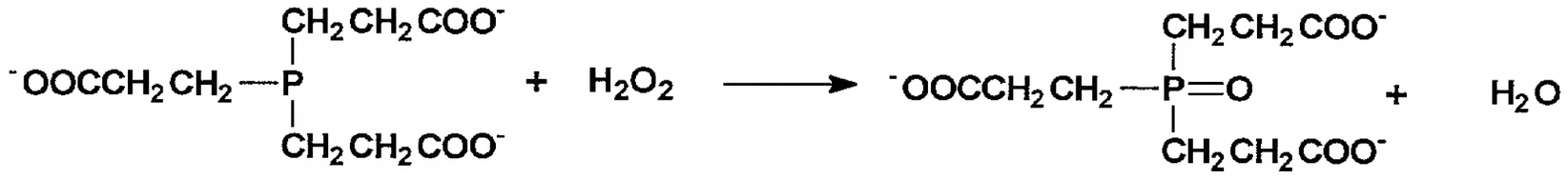
***b) Determination of $H_2O_2$***

This method is modified from a $H_2O_2$ assay reported recently (Han, J.C. *et al., Anal. Biochem. 234*:107 (1996)). The advantages of this modified $H_2O_2$ assay on 96-well plate include high throughput, excellent sensitivity (~1 μM), and the elimination of the need for a standard curve of $H_2O_2$ , which is problematic due to the labile chemical property of $H_2O_2$.

Aβ peptides were co-incubated with a $H_2O_2$-trapping reagent (Tris(2-carboxyethyl)-phosphine hydrochloride, TCEP, 100 μM) in PBS (pH 7.4 or 7.0) at 37°C for 30 mins. Then 5,5′-dithio-bis(2-nitrobenzoic acid) (DBTNB, 100 μM) was added to react with remaining TCEP. The product of this reaction has a characteristic absorbance maximum of 412 nm [18]. The assay was adapted to a 96-well format using a standard absorbance range (see FIG. 11).

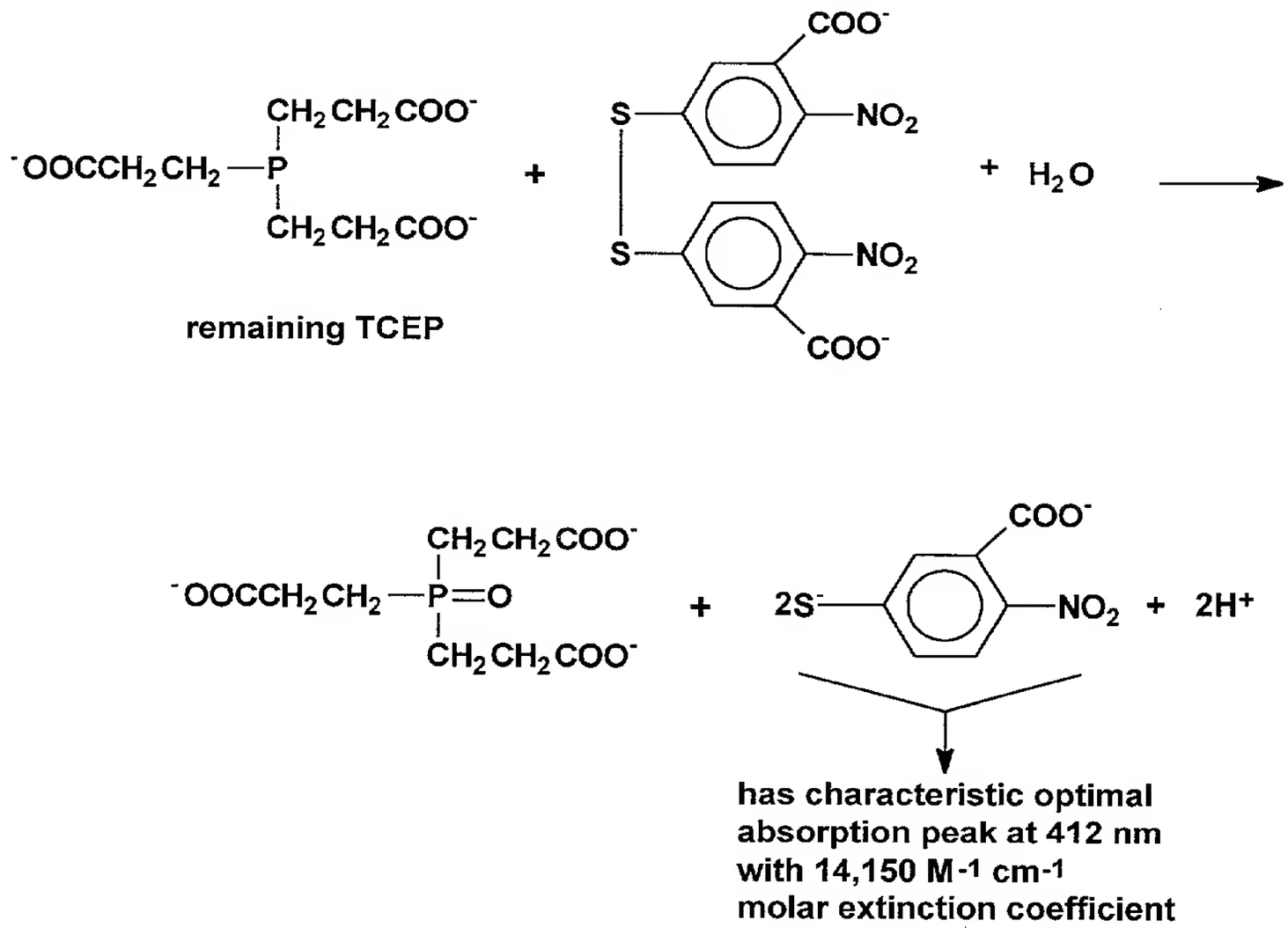
The chemical scheme for this novel method is:

**Scheme I:**

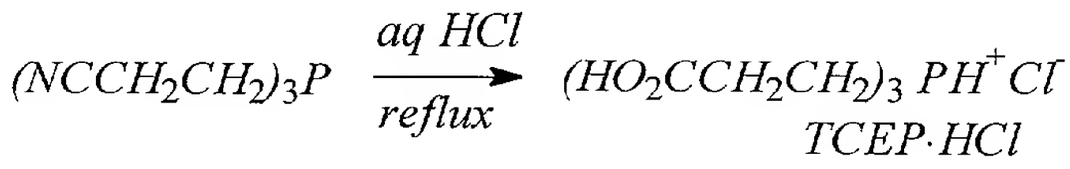



(TCEP) [Tris (2-carboxethyl) phosphine]

**Scheme II:**




TCEP•HCl was synthesized by hydrolyzing tris (2-cyno-ethyl) phosphine (purchased from Johnson-Mathey (Waydhill, MA)), in refluxing aqueous HCl (Burns, J.A. *et al.*, *J. Org. Chem.* *56*:2648 (1991)) as shown below.



In order to carry out the above-described assay in a 96-well plate, it was necessary to calculate the equivalent vertical pathlength of 2-nitro-5-thiobenzoic acid (TMB) in a 96-well plate. This determination was carried out essentially as described for $Cu^{+}$-BC and $Fe^{2+}$-BP in Example 5. The resulting absorbancies from the plate reader regress against absorbancies by a UV-vis spectrometer. The slope k from the linear regression line is equivalent to the vertical pathlength if the measurement is carried out on a plate. The results are:

| k | r2 |
|---|---|
| 0.875 | 1.00 |

The concentration of $H_2O_2$ can then be deduced from the difference in absorbance between the sample and the control (sample plus 1000 U/µl catalase)

$$[H_2O_2]\ (\mu M) = \frac{\Delta A\ (412nm)}{(2\ x\ 0.875\ x\ 14150)}$$

***c) Determination of OH·***

Determination of OH· was performed as described in Gutteridge *et al.* (*Biochim. Biophys. Acta 759*: 38-41 (1983)).

***d) Cu(I) generation by Aβ: influence of Zn(II) and pH***

Aβ (10 µM in PBS, pH 7.4 or 6.8, as shown) was incubated for 30 minutes (37°C) in the presence of Cu(II) 10 µM ± Zn(II) 10 µM). Cu(I) levels (n=3, ±SD) were assayed against a standard curve. These data indicate that the presence of Zn(II) can mediate the reduction of Cu(II) in a mildly acidic environment. The effects of zinc upon these reactions are strongly in evidence

but complex. Since the presence of 10 μM zinc will precipitate the peptide, it is clear that the peptide possesses redox activity even when it is not in the soluble phase, suggesting that cortical Aβ deposits will not be inert in terms of generating these highly reactive products. Cerebral zinc metabolism is deregulated in AD, and therefore levels of interstitial zinc may play an important role in adjusting the Cu(I) and $H_2O_2$ production generated by Aβ. The rat homologue of Aβ1→40 does not manifest the redox reactivity of the human equivalent. Insulin, a histidine-containing peptide that can bind copper aind zinc, exhibits no Cu(II) reduction.

### *e) Hydrogen peroxide production by Aβ species*

$AB_{1\text{-}42}$ (10 μM) was incubated for 1 hr at 37°C, pH 7.4 in ambient air (first bar), continuous argon purging (Ar), continuous oxygen enrichment ($O_2$) at pH 7.0 (7.0), or in the presence of the iron chelator desferioxamine (220 μM; DFO). Variant Aβ species (10 μM) were tested: $A\beta_{1\text{-}40}$ ($A\beta_{1\text{-}40}$), rat $A\beta_{1\text{-}40}$ (rAβ1→40), and scrambled Aβ1→40 ($sA\beta_{1\text{-}40}$) were incubated for 1 hr at 37°C, pH 7.4 in ambient air. Values (mean ±SD, n=3) represent triplicate samples minus values derived from control samples run under identical conditions in the presence of catalase (10 U/ml). The details of the experiment are as follows: Aβ peptides were co-incubated with a $H_2O_2$-trapping reagent (Tris(2-carboxyethyl)-phosphine hydrochloride, TCEP, 100 μM) in PBS (pH 7.4 or 7.0) at 37°C for 30 mins. Then 5,5′-dithio-bis(2-nitrobenzoic acid) (DTNB, 100 μM) was added to react with remaining TCEP, the product has a characteristic absorbance maximum of 412 nm. The assay was adapted to a 96-well format using a standard absorbance range.

## *Results and Discussion*

### *Aβ exhibits metal-dependent and independent redox activity*

Because Aβ was observed to be covalently linked by Cu, the ability of the peptide to reduce metals and generate hydroxyl radicals was studied. The bathocuproine and bathophenanthroline reduced metal assay technique employed by Multhaup *et al.* was used in order to determine that APP itself possesses a Cu(II) reducing site on its ectodomain (Multhaup, G., *et al.*, *Science 271*:1406 (1996)). It has been discovered that Aβ possesses a striking ability to reduce both Fe(III) to Fe(II), and Cu(II) to Cu(I), modulated by Zn(II) and pH (6.6-7,4) (FIG. 10). It is of great interest that the relative redox activity of the peptides studied correlates so well with their relative pathogenicity viz Aβ42>Aβ40>ratAβ in all redox assays studied. Since one of the caveats in using the reduced metals assay is that the detection agents can exaggerate the oxidation potential of Cu(II) or Fe (III), other redox products were explored by assays where no metal ion indicators were necessary. It was discovered that hydrogen peroxide was rapidly formed by Aβ species (FIG. 11). Thus, Aβ produces both $H_2O_2$ and reduced metals whilst also binding zinc. Structurally, this is difficult to envisage for a small peptide, but we have recently shown that Aβ is dimeric in physiological buffers. Since $H_2O_2$ and reduced metal species are produced in the same vicinity, these reaction products are liable to produce the highly toxic hydroxyl radical by Fenton chemistry, and the formation of hydroxyl radicals from these peptides has now been shown with the thiobarbituric acid assay. The formation of hydroxyl radicals correlates with the covalent polymerization of the peptide (FIG. 9) and can be blocked by hydroxyl scavengers. Thus the concentrations of Fe, Cu, Zn & H+ in the brain interstitial milliu could be important in facilitating precipitation and neurotoxicity for Aβ by direct (dimer formation) and indirect (Fe(II)/Cu(I) and $H_2O_2$ formation) mechanisms.

$H_2O_2$ production by Aβ explains the mechanism by which $H_2O_2$ has been described to mediate neurotoxicity (Behl, C., *et al.*, *Cell 77*:827 (1994)),

previously thought to be the product of cellular overproduction alone. Interestingly, the scrambled Aβ peptide produces appreciable $H_2O_2$ (FIG. 6) but no hydroxyl radicals. This is because the scrambled Aβ peptide is unable to reduce metal ions. Therefore, we conclude that what makes Aβ such a potent neurotoxin is its capacity to produce both reduced metal sand $H_2O_2$ at the same time. This "double whammy" can then produce hydroxyl radicals by the Fenton reaction, especially if the $H_2O_2$ is not rapidly removed from the vicinity of the peptide. Catalase and glutathione peroxidase are the principal means of catabolizing $H_2O_2$, and their levels are low in the brain, especially in AD, perhaps explaining the propensity of Aβ to accumulate in brain tissue.

FIG. 11 shows that the production of $H_2O_2$ is oxygen dependent, and further investigation has indicated that Aβ can spontaneously produce the superoxide radical ($O_2^-$) in the absence of metal ions. This property of Aβ is particularly exaggerated in the case of Aβ42, probably explaining why this peptide is more neurotoxic and more enriched than Aβ40 in amyloid. $O_2^-$ generation will be subject to spontaneous dismutation to generate $H_2O_2$, however, this is a relatively slow reaction, although it may account for the majority of the $H_2O_2$ detected in our Aβ assays. $O_2^-$ is reactive, and the function of superoxide dismutase (SOD) is to accelerate the dismutation to produce $H_2O_2$ which is then catabolized by catalase and peroxidases into oxygen and water. The most abundant form of SOD is Cu/Zn SOD, mutations of which cause another neurodegenerative disease, amyotrophic lateral sclerosis (Rosen, D., *et al.*, *Nature 364*:362 (1993)). Since Aβ, like Cu/Zn SOD, is a dimeric protein that binds Cu and Zn and reduces Cu(II) and Fe(III), we studied the $O_2^-$ dismutaion behavior of Aβ in the μsec time-scale using laser pulse photolysis. These experimetns have shown that Aβ exhibits Fe/Cu-dependent SOD-like activity with rate constants of dismutation at $\approx 10^8$ $M^{-1}$ $sec^{-1}$, which are strikingly similar to SOD. Hence, Aβ appears to be a good candidate to possess the same function as SOD, and therefore may function as an antioxidant. This may explain why oxidative stresses cause it to be released by cells (Frederikse, P.H., *et al.*, *Journal of*

*Biological Chemistry 271*: 10169 (1996)). However, if $A\beta_{1-42}$ is involved in the reaction to oxidative stress, or if the $H_2O_2$ clearance is compromised at the cellular level, Aβ will accumulate, recruiting more $O_2$ and producing more $O_2^-$ leading to a vicious cycle and localizing tissue Peroxidation damage and protein cross-linking.

## *Example 3*

### *Cell Culture and Cytotoxic Assays*

Several different assays may be utilized to determine whether a candidate pharmacological agent identified by any of the above-summarized assays is capable of altering the neurotoxicity of Aβ. The first is the MTT assay, which measures the reduction of 3-(4,5-dimethylthiazol-2-yl)-2,5, diphenyl tetrazolium bromide (MTT) to a colored formazon (Hansen *et al.*, 1989). A second cytotoxic assay is the release of lactic dehydrogenase (LDH) from cells, a measurement routinely used to quantitate cytotoxicity in cultured CNS cells (Choi, 1987). While MTT measures primarily early redox changes within the cell reflecting the integrity of the electron transport chain, the release of LDH is thought to be through cell lysis. A third assay is visual counting in conjunction with trypan blue exclusion. Yet another assay is the Live/Dead EukoLight Viability/Cytotoxicity Assay (Molecular Probes, Inc., Eugene, OR).

Having now fully described this invention, it will be understood by those of skill in the art that it can be performed within any wide range of equivalent modes of operation as well as other parameters without affecting the scope of the invention or any embodiment thereof.

All patents and publications cited in the present specification are incorporated by reference herein in their entirety.

## *What Is Claimed Is:*

1. A method for the identification of an agent to be used in the treatment of AD, wherein said agent is capable of altering the production of Cu(I) by Aβ, said method comprising:

(a) adding Cu(II) to a first Aβ sample;

(b) allowing said first sample to incubate for an amount of time sufficient to allow said first sample to generate Cu(I);

(c) adding Cu(II) to a second Aβ sample, said second sample additionally comprising a candidate pharmacological agent;

(d) allowing said second sample to incubate for the same amount of time as said first sample;

(e) determining the amount of Cu(I) produced by said first sample and said second sample; and

(f) comparing the amount of Cu(I) produced by said first sample to the amount of Cu(I) produced by said second sample;

whereby a difference in the amount of Cu(I) produced by said first sample as compared to said second sample indicates that said candidate pharmacological agent has altered the production of Cu(I) by Aβ.

2. The method of claim 1, wherein the amount of Cu(I) present in said first and said second sample is determined by

(a) adding a complexing agent to said first and said second sample, wherein said complexing agent is capable of combining with Cu(I) to form a complex compound, wherein said complex compound has an optimal visible absorption wavelength;

(b) measuring the absorbancy of said first and said second sample; and

(c) calculating the concentration of Cu(I) in said first and said second sample using the absorbancy obtained in step (b).

3. The method of claim 2, wherein said complexing agent is bathocuproinedisulfonic anion.

4. The method of claim 2 or claim 3, wherein said method is performed in a microtiter plate, and the absorbancy measurement is performed by a plate reader.

5. The method of claim 4, wherein two or more different test candidate agents are simultaneously evaluated for an ability to alter the production of Cu(I) by Aβ.

6. The method of claim 1, wherein said first Aβ sample of step 1(a) and said second Aβ sample of step 1(c) is a biological sample.

7. The method of claim 6, wherein said biological sample is CSF.

8. A method for the identification of an agent to be used in the treatment of AD, wherein said agent is capable of altering the production of Fe(II) by Aβ, said method comprising:

(a) adding Fe(III) to a first Aβ sample;

(b) allowing said first sample to incubate for an amount of time sufficient to allow said first sample to generate Fe(II);

(c) adding Fe(III) to a second Aβ sample, said second sample additionally comprising a candidate pharmacological agent;

(d) allowing said second sample to incubate for the same amount of time as said first sample;

(e) determining the amount of Fe(II) produced by said first sample and said second sample; and

(f) comparing the amount of Fe(II) present in said first sample to the amount of Fe(II) present in said second sample;

whereby a difference in the amount of Fe(II) present in said first sample as compared to said second sample indicates that said candidate pharmacological agent has altered the production of Fe(II) by Aβ.

9. The method of claim 8, wherein the amount of Fe(II) present in said first and said second sample is determined by

(a) adding a complexing agent to said first and said second sample, wherein said complexing agent is capable of combining with Fe(II) to form a complex compound, wherein said complex compound has an optimal visible absorption wavelength;

(b) measuring the absorbancy of said first and said second sample; and

(c) calculating the concentration of Fe(II) in said first and said second sample using the absorbancy obtained in step (b).

10. The method of claim 9, wherein said complexing agent is bathophenanthrolinedisulfonic (BP) anion.

11. The method of claim 9 or claim 10, wherein said method is performed in a microtiter plate, and the absorbancy measurement is performed by a plate reader.

12. The method of claim 11, wherein two or more different test candidate agents are simultaneously evaluated for an ability to alter the production of Fe(II) by Aβ.

13. The method of claim 8, wherein said first Aβ sample of step 1(a) and said second Aβ sample of step 1(c) is a biological sample.

14. The method of claim 13, wherein said biological sample is CSF.

15. A method for the identification of an agent to be used in the treatment of AD, wherein said agent is capable of altering the production of $H_2O_2$ by Aβ, said method comprising:

(a) adding Cu(II) or Fe(III) to a first Aβ sample;

(b) allowing said first sample to incubate for an amount of time sufficient to allow said first sample to generate $H_2O_2$;

(c) adding Cu(II) or Fe(III) to a second Aβ sample, said second sample additionally comprising a candidate pharmacological agent;

(d) allowing said second sample to incubate for the same amount of time as said first sample;

(e) determining the amount of $H_2O_2$ produced by said first sample and said second sample; and

(f) comparing the amount of $H_2O_2$ present in said first sample to the amount of $H_2O_2$ present in said second sample;

whereby a difference in the amount of $H_2O_2$ present in said first sample as compared to said second sample indicates that said candidate pharmacological agent has altered the production of $H_2O_2$ by Aβ.

16. The method of claim 15, wherein the Aβ samples of steps (a) and step (b) are a biological fluid.

17. The method of claim 16, wherein said biological fluid is CSF.

18. The method of claim 15, wherein the determination of step (e) of the amount of $H_2O_2$ present in said first and said second sample is determined by

(a) adding catalase to a first aliquot of said first sample obtained in step (a) of claim 1 in an amount sufficient to break down all of the $H_2O_2$ generated by said sample;

(b) adding TCEP, in an amount sufficient to capture all of the $H_2O_2$ generated by said samples, to

(i) said first aliquot

(ii) a second aliquot of said first sample obtained in step (a) of claim 1; and

(iii) said second sample obtained in step (b) of claim 1;

(c) incubating the samples obtained in step (b) for an amount of time sufficient to allow the TCEP to capture all of the $H_20_2$;

(d) adding DTNB to said samples obtained in step (c);

(e) incubating said samples obtained in step (d) for an amount of time sufficient to generate TMB;

(f) measuring the absorbancy at 412 nm of said samples obtained in step (e); and

(g) calculating the concentration of $H_2O_2$ in said first and said second sample using the absorbancies obtained in step (f).

19. The method of claim 18, wherein said method is performed in a microtiter plate, and the absorbancy measurement is performed by a plate reader.

20. The method of claim 19, wherein two or more different test candidate agents are simultaneously evaluated for an ability to alter the production of $H_2O_2$ by Aβ.

21. A method for the identification of an agent to be used in the treatment of AD, wherein said agent is capable of decreasing the production of $O_2$- by Aβ, said method comprising:

(a) adding Aβ and to a first buffer sample having an $O_2$ tension greater than 0;

(b) allowing said first sample to incubate for an amount of time sufficient to allow said first sample to generate $O_2$-;

(c) adding Aβ and a candidate pharmacological agent to a second buffer sample having an $O_2$ tension greater than 0;;

(d) allowing said second sample to incubate for the same amount of time as said first sample;

(e) determining the amount of $O_2$- produced by said first sample and said second sample; and

(f) comparing the amount of $O_2$- present in said first sample to the amount of $O_2$- present in said second sample;

whereby a difference in the amount of $O_2$- present in said first sample as compared to said second sample indicates that said candidate pharmacological agent has altered the production of $O_2$- by Aβ.

22. The method of claim 21, wherein said Aβ is $A\beta_{1-42}$.

23. The method of claim 21, wherein the determination of the amount of $O_2$- present in said samples is accomplished by measuring the absorbancy of the sample at about 250 nm.

24. A method for the identification of an agent to be used in the treatment of AD, wherein said agent is capable of interfering with the interaction of $O_2$ and Aβ to produce $O_2$-, without interfering with the SOD-like activity of Aβ, said method comprising:

(a) identifying an agent capable of decreasing the production of $O_2$- by Aβ; and

(b) determining the ability of said agent to alter the SOD-like activity of Aβ.

25. The method of claim 24, wherein the determination of the ability of said agent to alter the SOD-like activity of Aβ is made by determining whether Aβ is capable of catalytically producing Cu(I), Fe(II) or $H_2O_2$.

26. A method for the identification of an agent to be used in the treatment of AD, wherein said agent is capable of reducing the toxicity of Aβ, said method comprising:

(a) adding Aβ to a first cell culture;

(b) adding Aβ to a second cell culture, said second cell culture additionally containing a candidate pharmacological agent;

(c) determining the level of neurotoxicity of Aβ in said first and said second samples; and

(d) comparing the level of neurotoxicity of Aβ in said first and said second samples,

whereby a lower neurotoxicity level in said second sample as compared to said first sample indicates that said candidate pharmacological agent has reduced the neurotoxicity of Aβ, and is thereby capable of being used to treat AD.

27. The method of claim 26, wherein the neurotoxicity of Aβ is determined by using an MTT assay.

28. The method of claim 26, wherein the neurotoxicity of Aβ is determined by using an LDH release assay.

29. The method of claim 26, wherein the neurotoxicity of Aβ is determined by using a Live/Dead assay.

30. The method of claim 26, wherein said cells are rat cancer cells.

31. The method of claim 26, wherein said cells are rat primary frontal neuronal cells.

32. A kit for determining whether an agent is capable of altering the production of Cu(I) by Aβ which comprises a carrier means being

compartmentalized to receive in close confinement therein one or more container means wherein

(a) the first container means contains a peptide comprising Aß peptide;

(b) a second container means contains a Cu(II) salt; and

(c) a third container means contains BC anion.

33. The kit of claim 32, wherein said Aβ peptide is present as a solution in an aqueous buffer or a physiological solution, at a concentration above about 10 $\mu$M.

34. A kit for determining whether an agent is capable of altering the production of Fe(II) by Aβ which comprises a carrier means being compartmentalized to receive in close confinement therein one or more container means wherein

(a) the first container means contains a peptide comprising Aß peptide;

(b) a second container means contains an Fe(III) salt; and

(c) a third container means contains BP anion.

35. The kit of claim 34, wherein said Aβ peptide is present as a solution in an aqueous buffer or a physiological solution, at a concentration above about 10 $\mu$M.

36. A kit for determining whether an agent is capable of altering the production of $H_2O_2$ by Aβ which comprises a carrier means being compartmentalized to receive in close confinement therein one or more container means wherein

(a) the first container means contains a peptide comprising Aß peptide;

(b) a second container means contains a Cu(II) salt;

(c) a third container means contains TCEP; and

(d) a fourth container means contains DTNB. .

37. The kit of claim 36, wherein said Aβ peptide is present as a solution in an aqueous buffer or a physiological solution, at a concentration above about 10 $\mu$M.

38. A method for the identification of an agent to be used in the treatment of AD, wherein said agent is capable of inhibiting redox-reactive metal -mediated crosslinking Aβ, said method comprising:

(a) adding a redox-reactive metal to a first Aβ sample;

(b) allowing said first sample to incubate for an amount of time sufficient to allow Aβ crosslinking;

(c) adding said redox-reactive metal to a second Aβ sample, said second sample additionally comprising a candidate pharmacological agent;

(d) allowing said second sample to incubate for the same amount of time as said first sample;

(e) removing an aliquot from each of said first and said second sample; and

(f) determining presence or absence of crosslinking in said first and second samples,

whereby an absence of Aβ crosslinking in said second sample as compared to said first sample indicates that said candidate pharmacological agent has inhibited Aβ crosslinking.

39. The method of claim 38, wherein at step (f), a western blot analysis is performed to determine the presence or absence of crosslinking in the first and the second sample.

# Methods for the Identification of Agents for Use in the Treatment of Alzheimer's Disease

## *Abstract*

The invention relates to methods for the identification of candidate pharmacological agents to be used in the treatment of Alzheimer's disease and related pathological conditions.

# Declaration for Patent Application

Docket Number: 0609.4350000

As a below named inventor, I hereby declare that:

My residence, post office address and citizenship are as stated below next to my name.

I believe I am the original, first and sole inventor (if only one name is listed below) or an original, first and joint inventor (if plural names are listed below) of the subject matter that is claimed and for which a patent is sought on the invention entitled Method for the Identification of Agents for Use in the Treatment of Alzheimer's Disease, the specification of which is attached hereto unless the following box is checked:

☒ was filed on March 11, 1997 ;
as United States Application Number or PCT International Application Number 08/816,122 ; and
was amended on _____ (if applicable).

I hereby state that I have reviewed and understand the contents of the above identified specification, including the claims, as amended by any amendment referred to above.

I acknowledge the duty to disclose information that is material to patentability as defined in 37 C.F.R. § 1.56.

I hereby claim foreign priority benefits under 35 U.S.C. § 119(a)-(d) or § 365(b) of any foreign application(s) for patent or inventor's certificate, or § 365(a) of any PCT international application, which designated at least one country other than the United States listed below, and have also identified below any foreign application for patent or inventor's certificate, or PCT international application having a filing date before that of the application on which priority is claimed.

| Prior Foreign Application(s) | | | Priority Claimed |
|---|---|---|---|
| _____ (Application No.) | _____ (Country) | _____ (Day/Month/Year Filed) | ☐ Yes ☐ No |
| _____ (Application No.) | _____ (Country) | _____ (Day/Month/Year Filed) | ☐ Yes ☐ No |

I hereby claim the benefit under 35 U.S.C. § 119(e) of any United States provisional application(s) listed below.

| | |
|---|---|
| _____ (Application No.) | _____ (Filing Date) |
| _____ (Application No.) | _____ (Filing Date) |

I hereby claim the benefit under 35 U.S.C. § 120 of any United States application(s), or under § 365(c) of any PCT international application designating the United States, listed below and, insofar as the subject matter of each of the claims of this application is not disclosed in the prior United States or PCT international application in the manner provided by the first paragraph of 35 U.S.C. § 112, I acknowledge the duty to disclose information that is material to patentability as defined in 37 C.F.R. § 1.56 that became available between the filing date of the prior application and the national or PCT international filing date of this application.

| | | |
|---|---|---|
| _____ (Application No.) | _____ (Filing Date) | _____ (Status - patented, pending, abandoned) |
| _____ (Application No.) | _____ (Filing Date) | _____ (Status - patented, pending, abandoned) |

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I hereby declare that all statements made herein of my own knowledge are true and that all statements made on information and belief are believed to be true; and further that these statements were made with the knowledge that willful false statements and the like so made are punishable by fine or imprisonment, or both, under Section 1001 of Title 18 of the United States Code and that such willful false statements may jeopardize the validity of the application or any patent issued thereon.

| | |
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| Citizenship | |
| Post Office Address | |
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SKGF Rev 4/3/96

(Supply similar information and signature for subsequent joint inventors, if any)

# POWER OF ATTORNEY FROM ASSIGNEE

The General Hospital Corporation, a corporation of Massachusetts, having a principal place of business at Fruit Street, Boston, Massachusetts 02114, is assignee of the entire right, title and interest for the United States of America (as defined in 35 U.S.C. § 100), by reason of an Assignment to the Assignee executed on √July 11, June 25, June 25 and June 25, 1997, respectively of an invention known as Method for the Identification of Agents for Use in the Treatment of Alzheimer's Disease (Attorney Docket No. 0609.4350000), which is disclosed and claimed in a patent application of the same title by the inventors (1) Ashley I. BUSH, (2) Xudong HUANG, (3) Craig S. ATWOOD and (4) Rudolph E. TANZI (said application filed on March 11, 1997 at the U.S. Patent and Trademark Office, having Application Number 08/816,122).

RULE Reg No 32,893

The Assignee hereby appoints the following U.S. attorneys to prosecute this application and any continuation, divisional, continuation-in-part, or reissue application thereof, and to transact all business in the U.S. Patent and Trademark Office connected therewith: Robert Greene Sterne, Registration No. 28,912; Edward J. Kessler, Registration No. 25,688; Jorge A. Goldstein, Registration No. 29,021; Samuel L. Fox, Registration No. 30,353; David K.S. Cornwell, Registration No. 31,944; Robert W. Esmond, Registration No. 32,893; Tracy-Gene G. Durkin, Registration No. 32,831; Michele A. Cimbala, Registration No. 33,851; Michael B. Ray, Registration No. 33,997; Robert E. Sokohl, Registration No. 36,013; Eric K. Steffe, Registration No. 36,688; and Andrea G. Reister, Registration No. 36,253. The Assignee hereby grants said attorneys the power to insert on this Power of Attorney any further identification that may be necessary or desirable in order to comply with the rules of the U.S. Patent and Trademark Office.

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FOR: The General Hospital Corporation

SIGNATURE: _____

BY: DAVID J. GLASS, Ph.D.
ASSOCIATE DIRECTOR FOR PATENTS

TITLE: OFFICE OF TECHNOLOGY AFFAIRS

DATE: 7/11/97

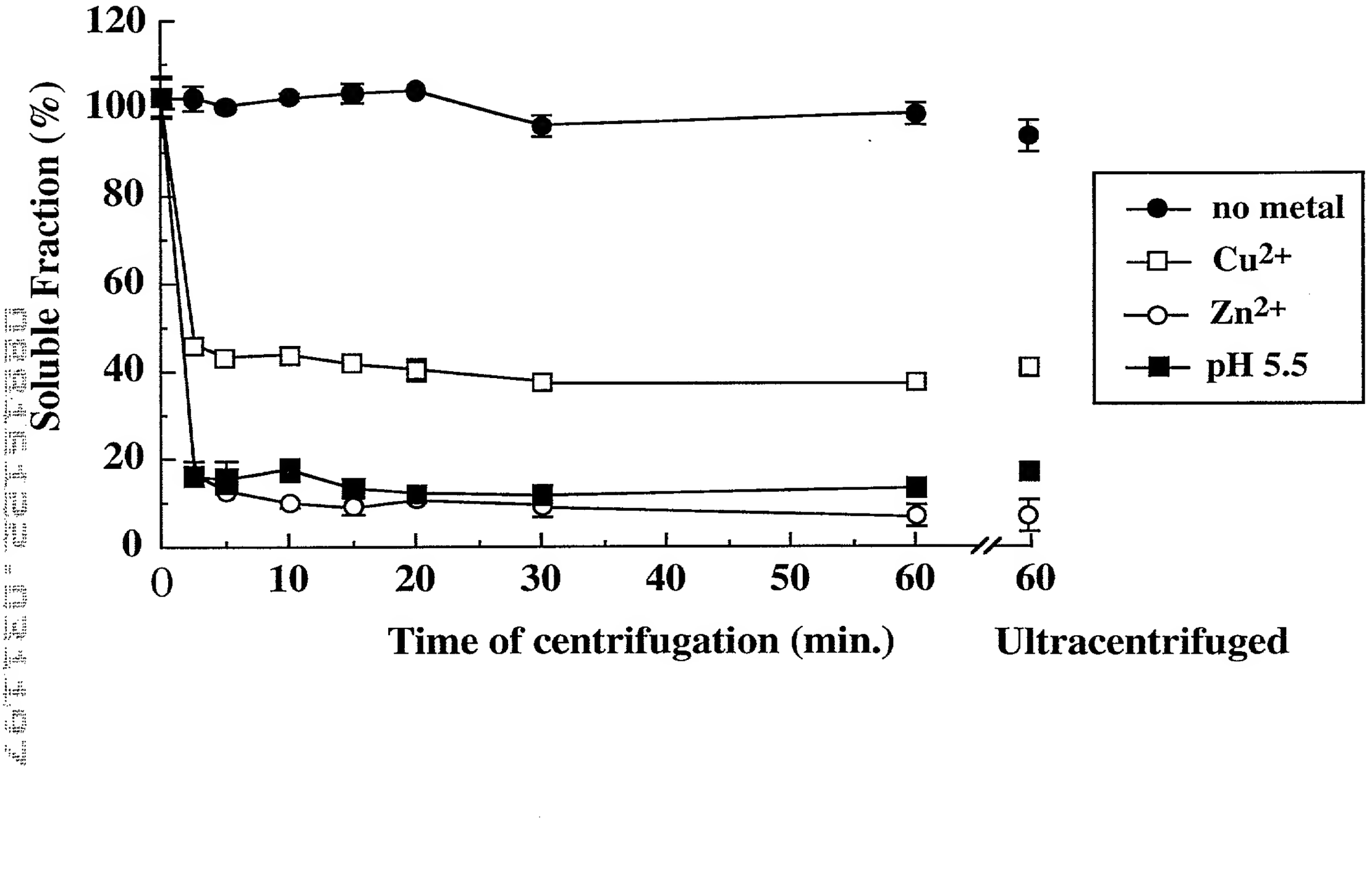


Fig 1.

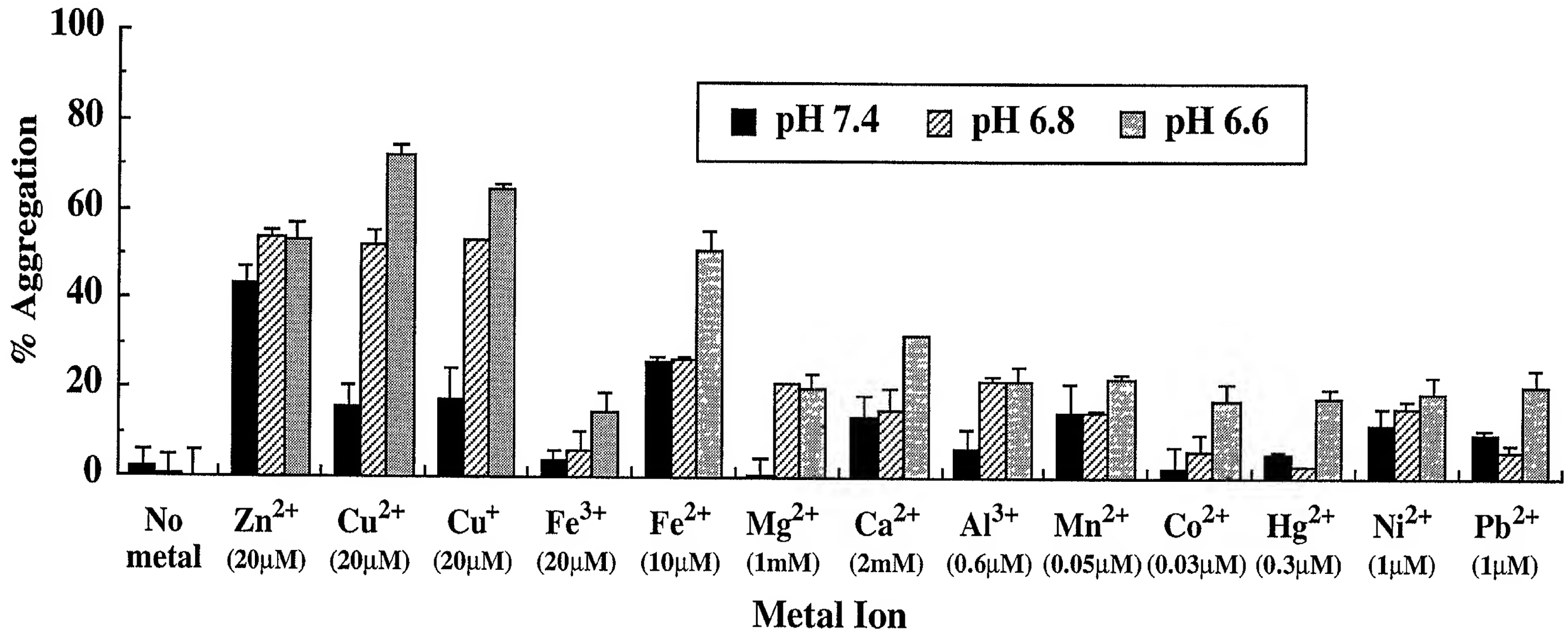


Fig 2a.

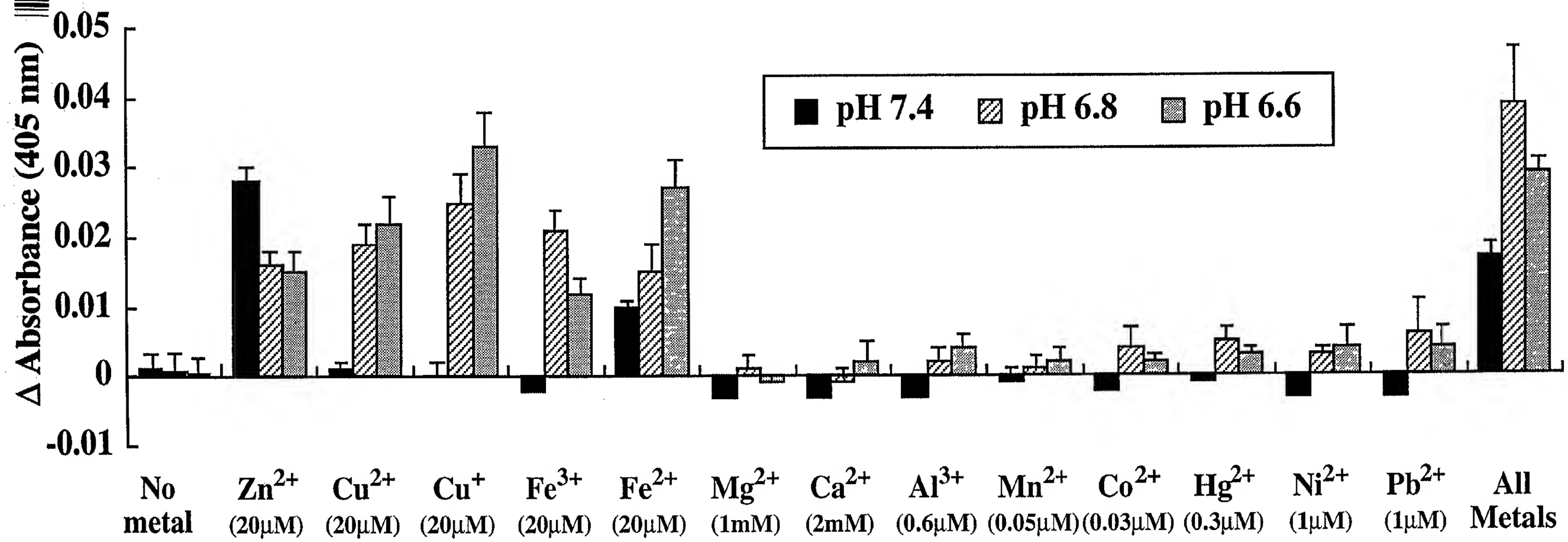


Fig 2b.

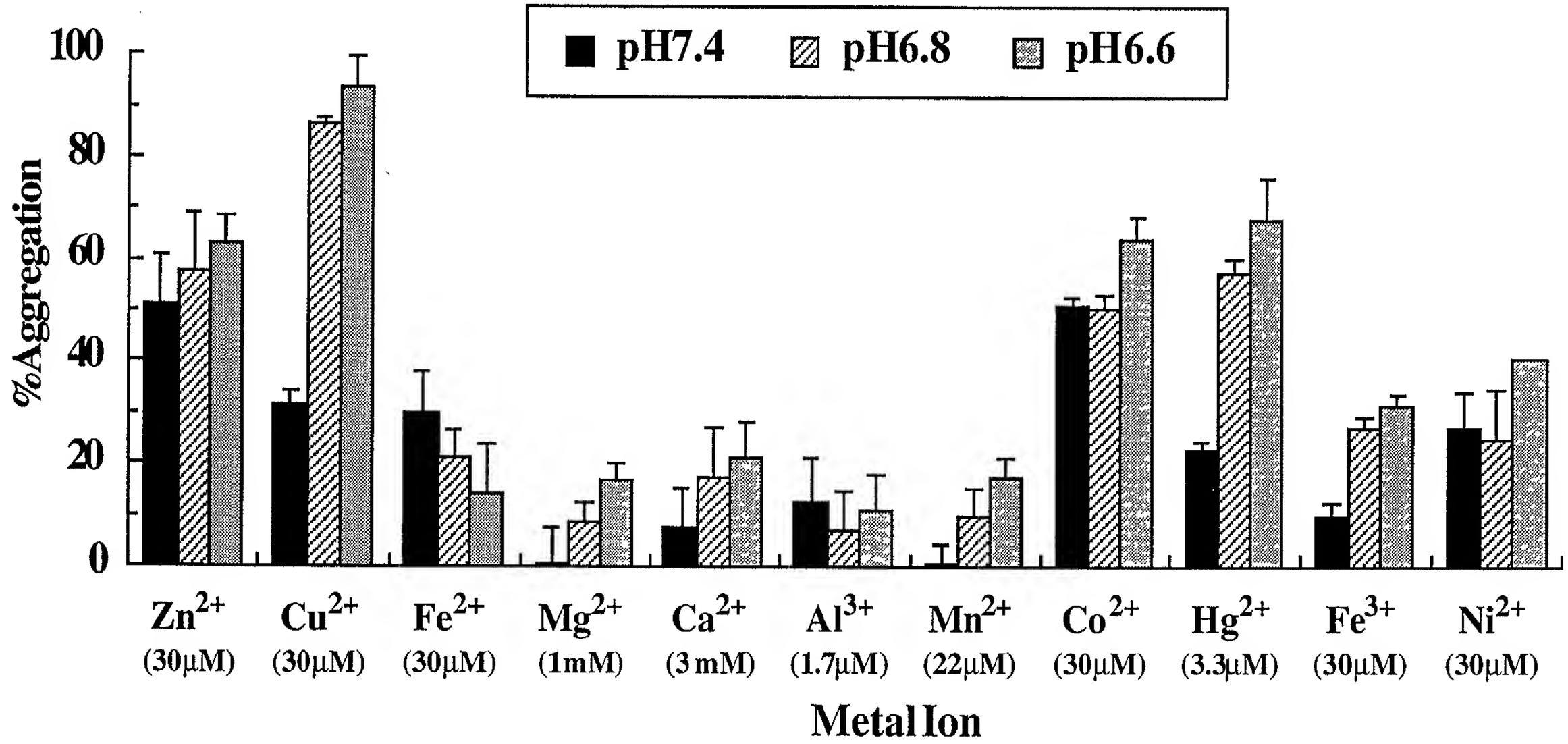


Fig 2c.

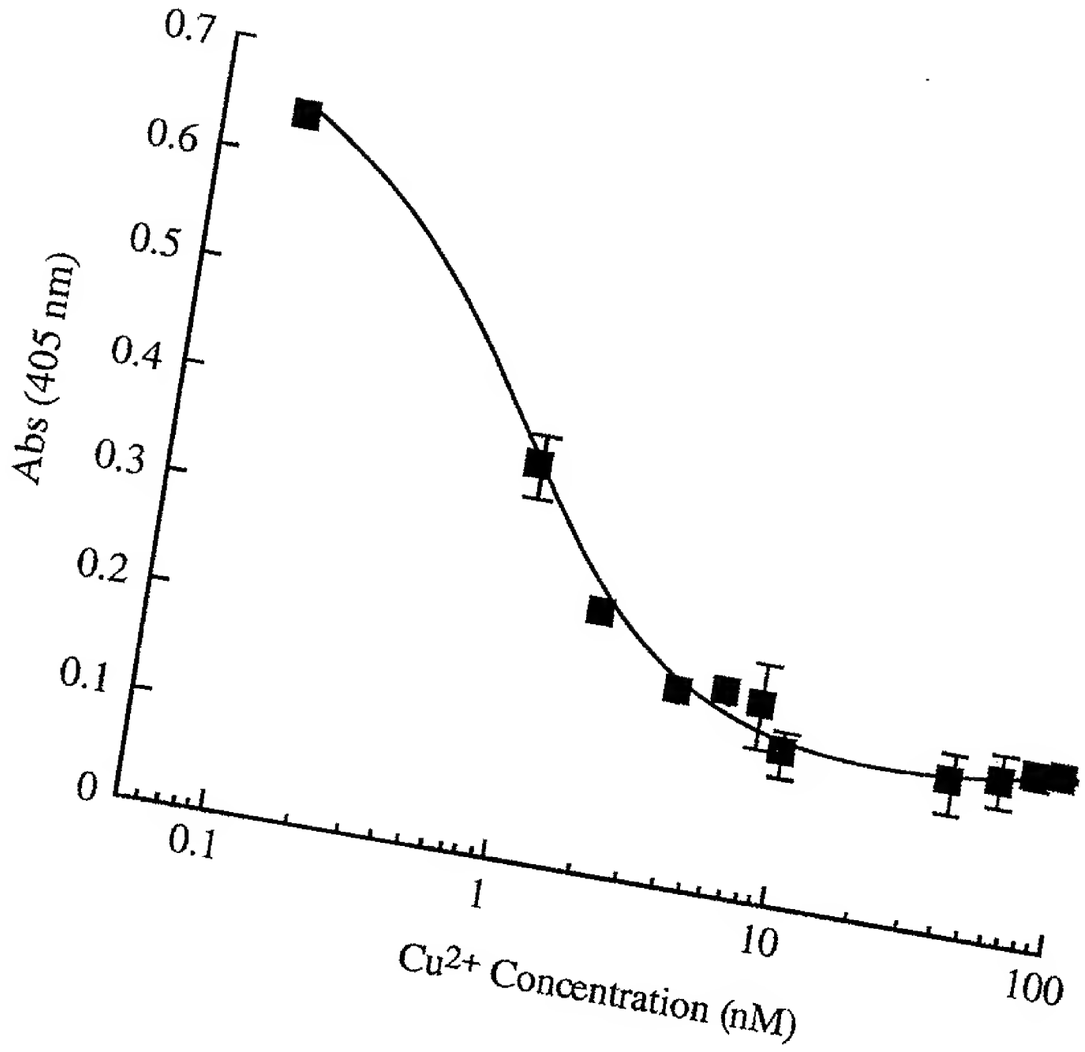


Fig 3

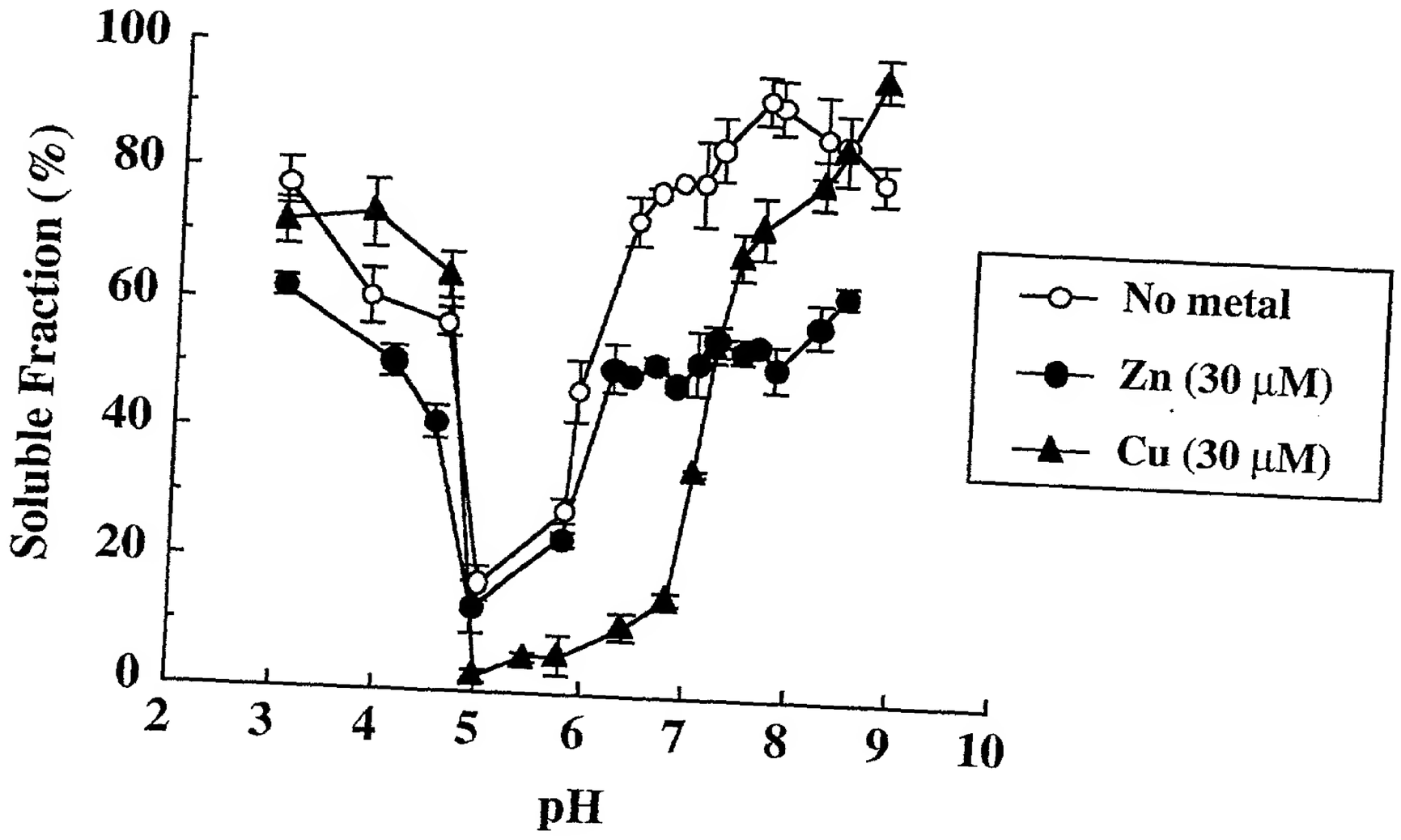


Fig 4a

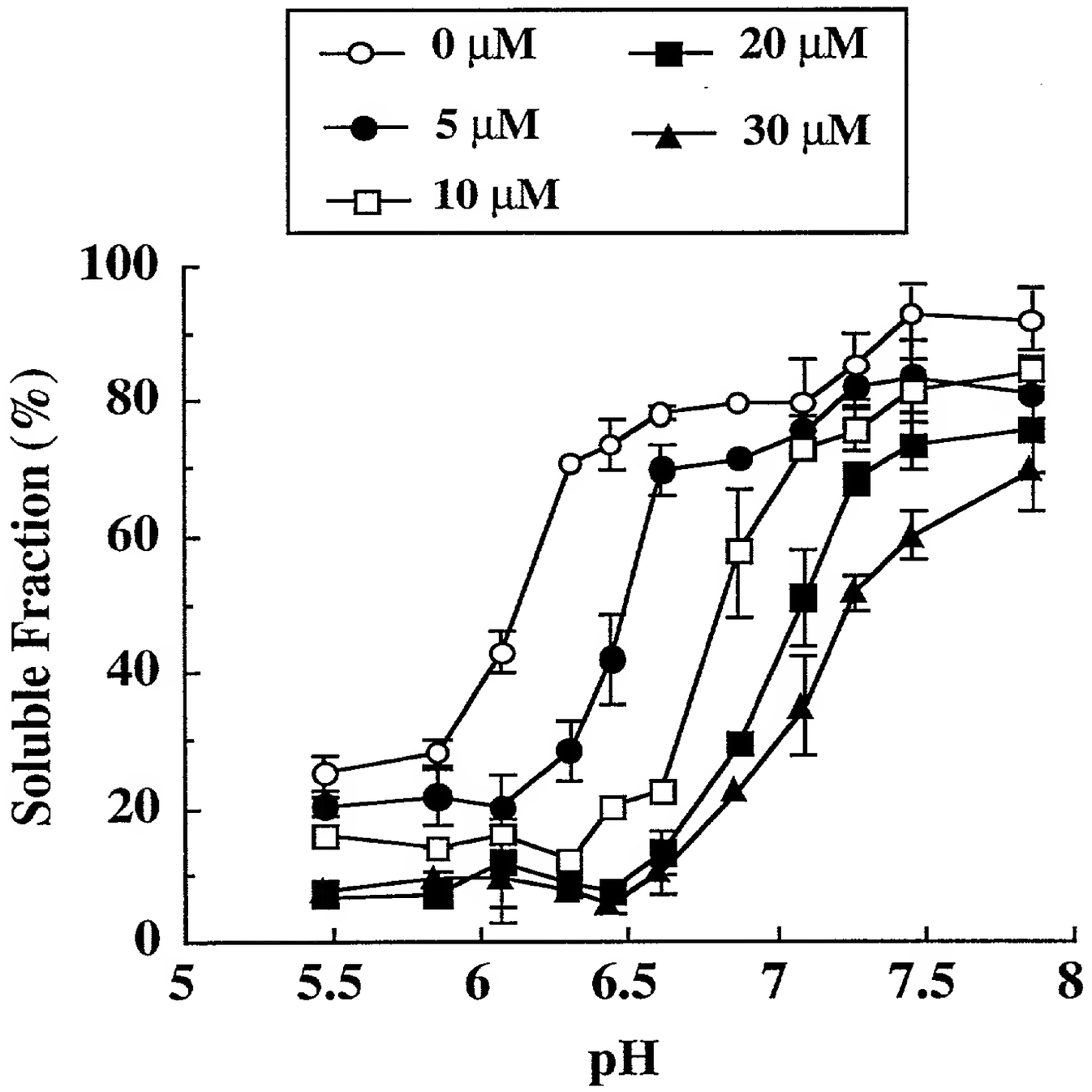


Fig 4b.

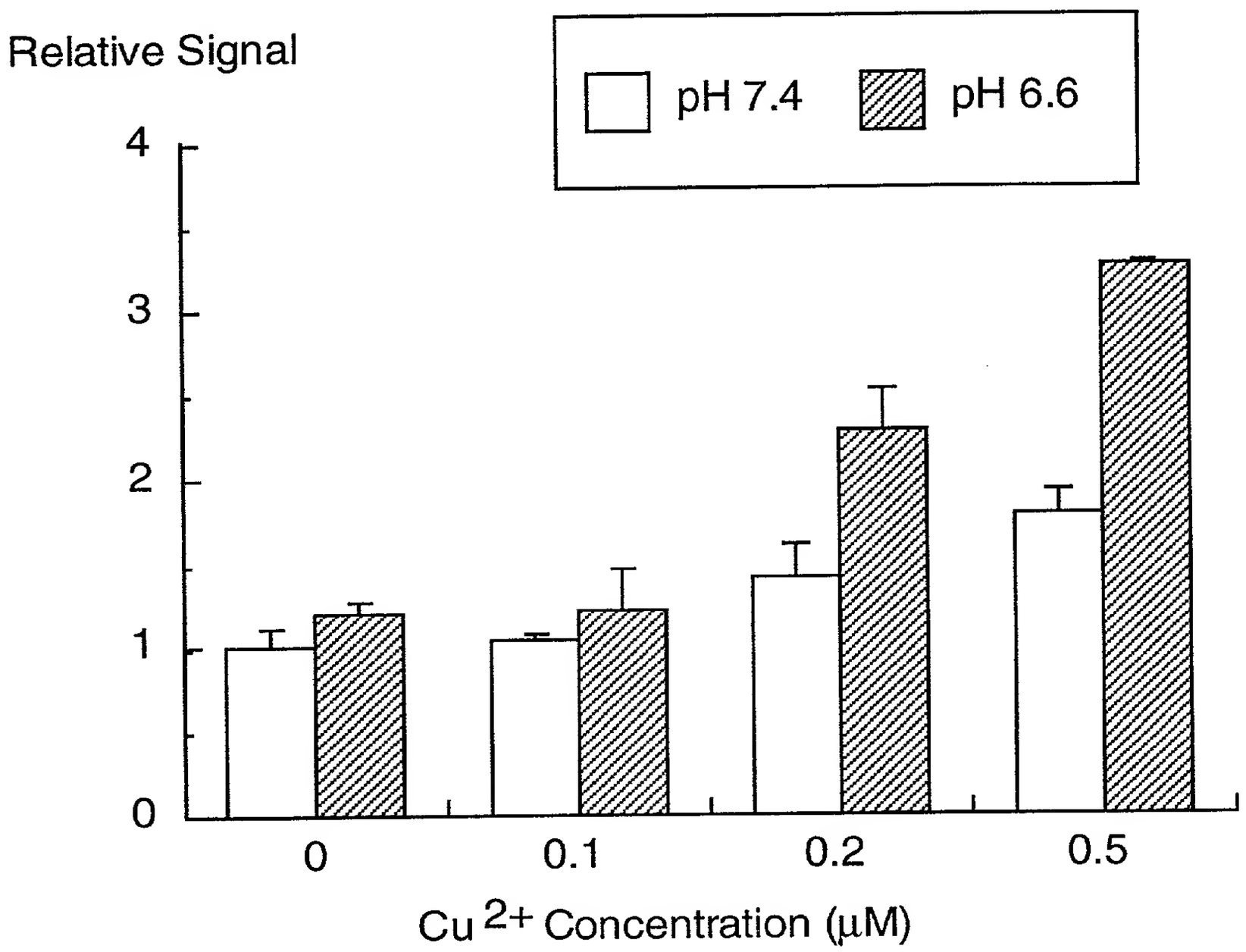


Fig 4c

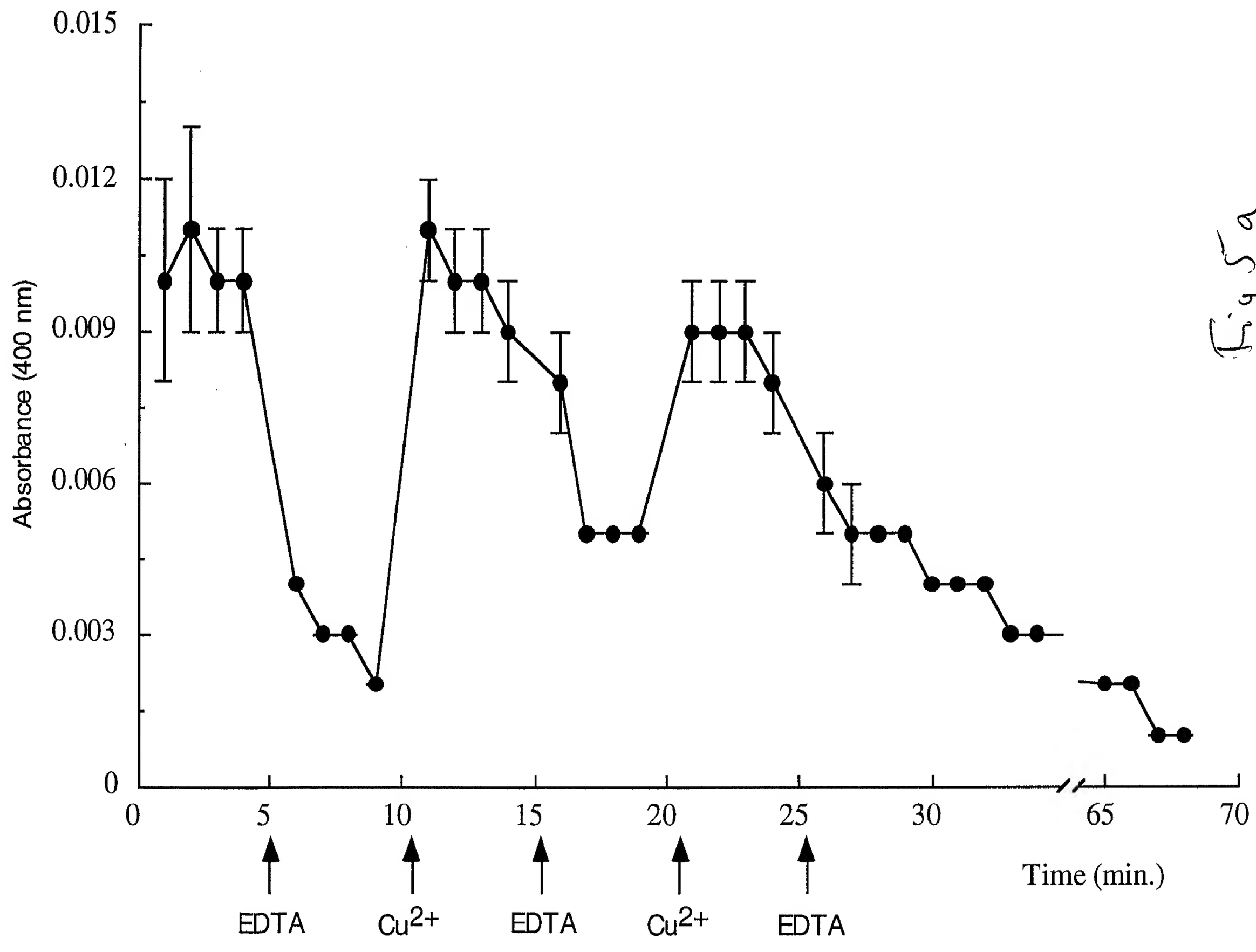


Fig 5a.

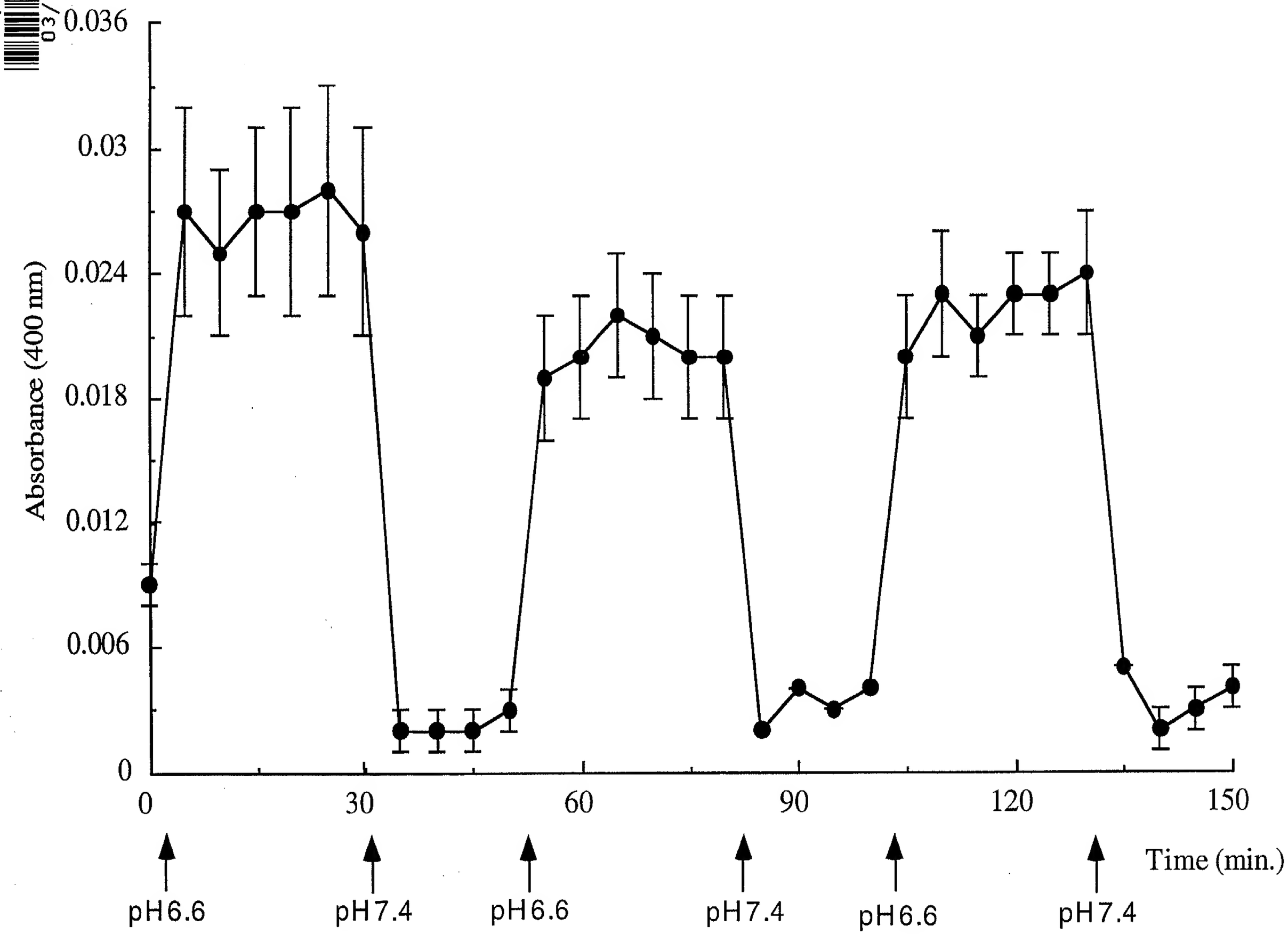


Fig 5-6

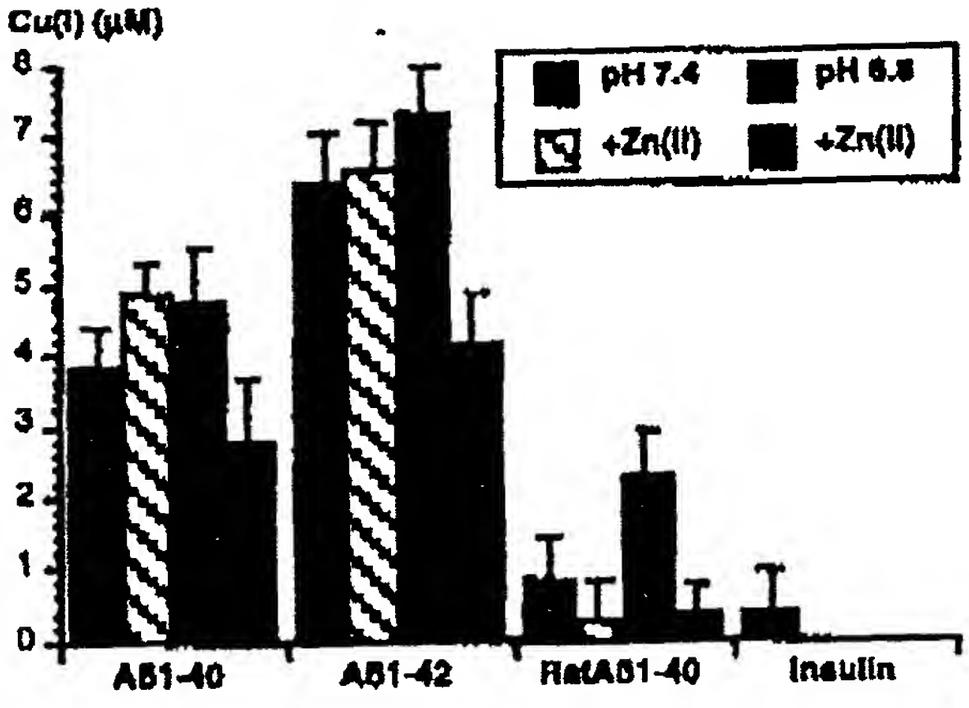


Fig. 6

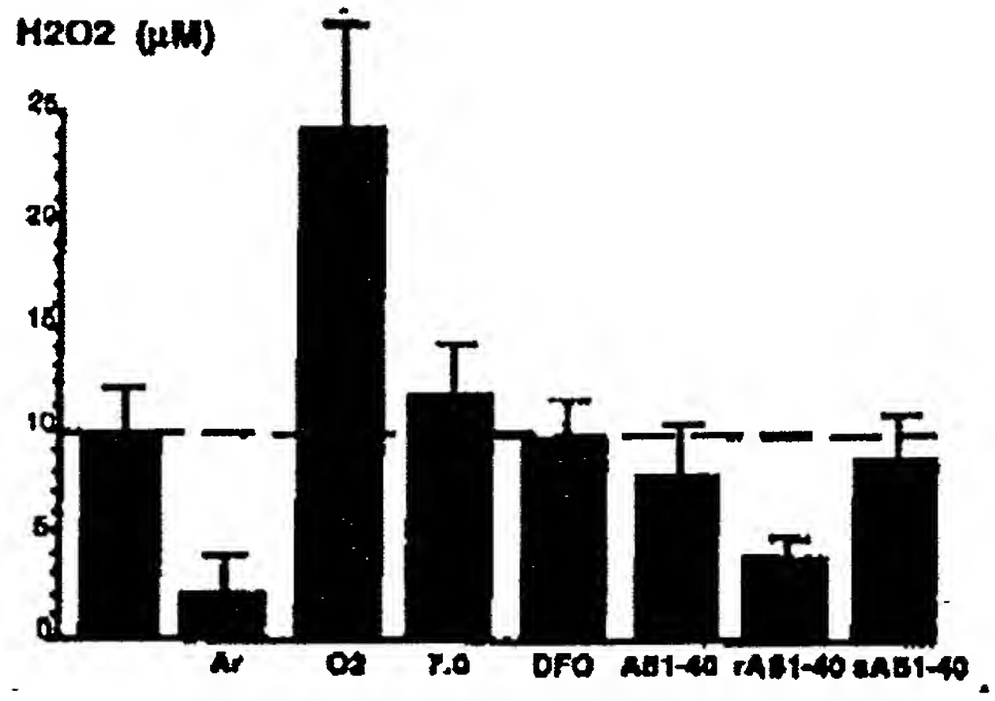


Fig. 7

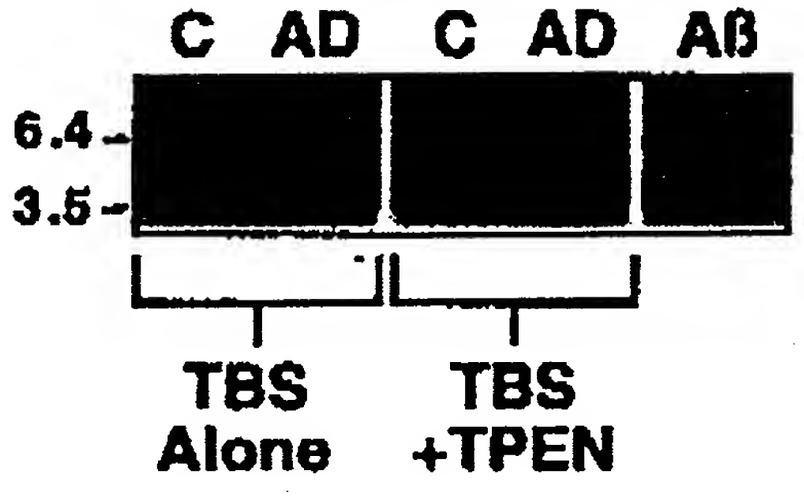


Fig. 8

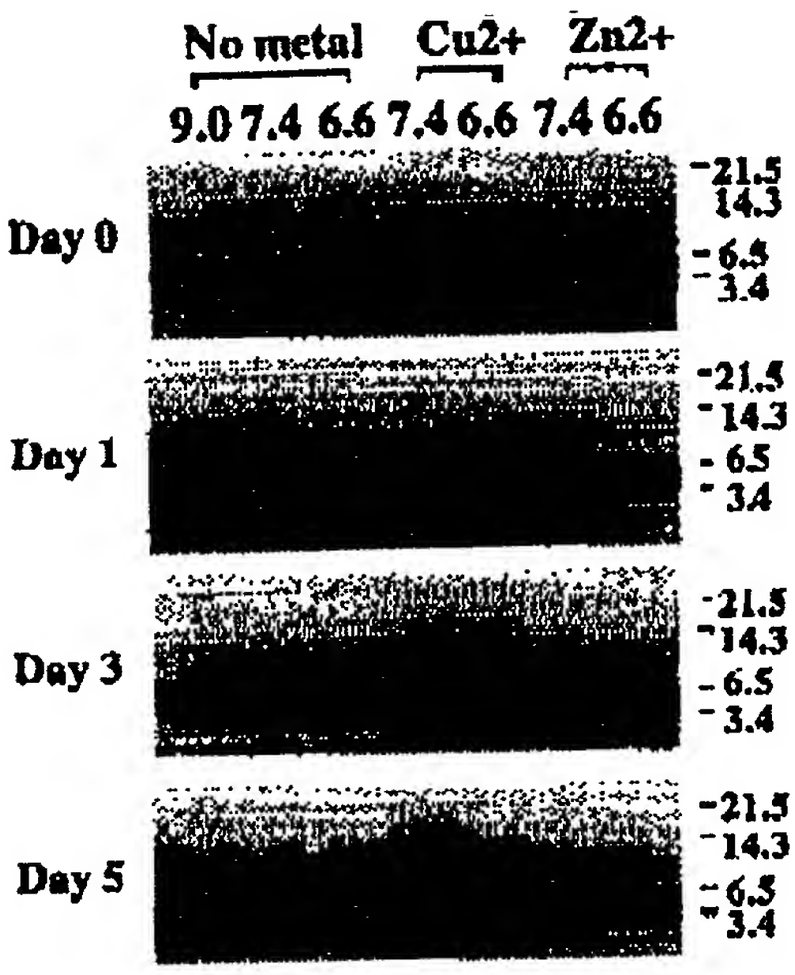


Fig. 9

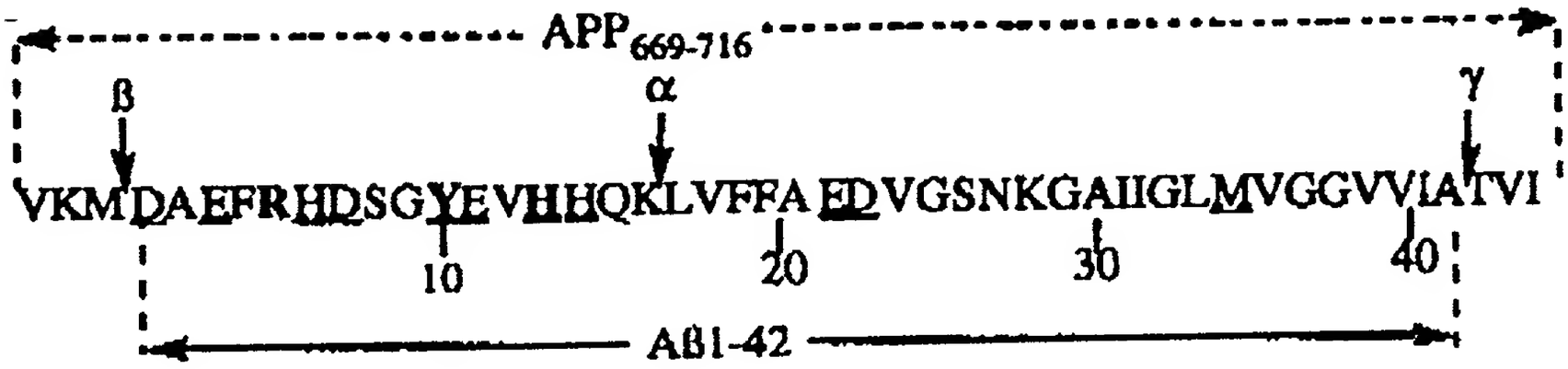


Fig. 10

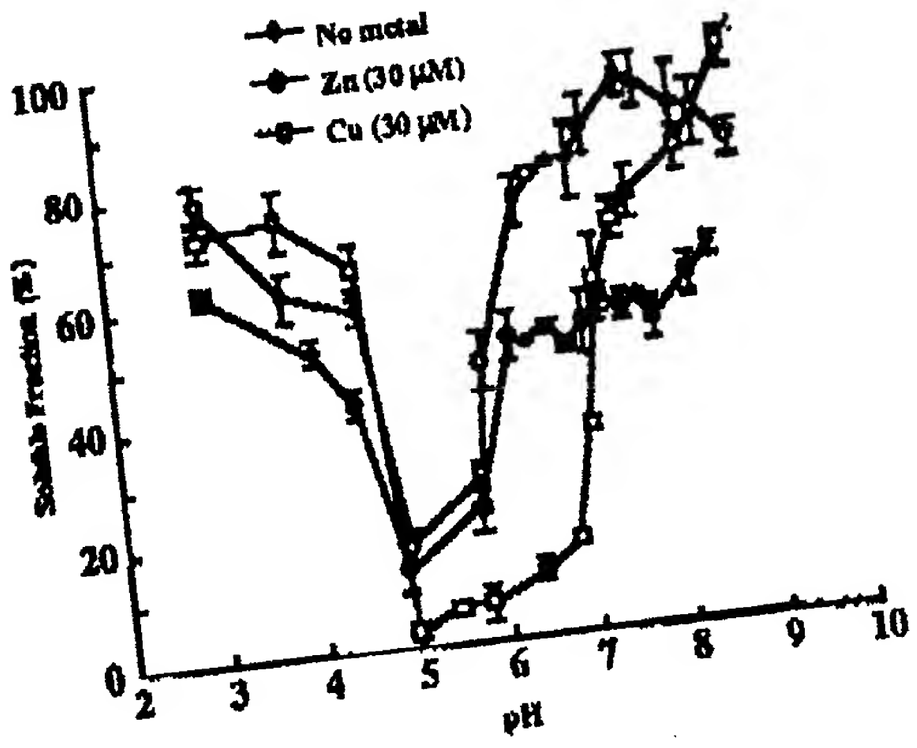


Fig. 11

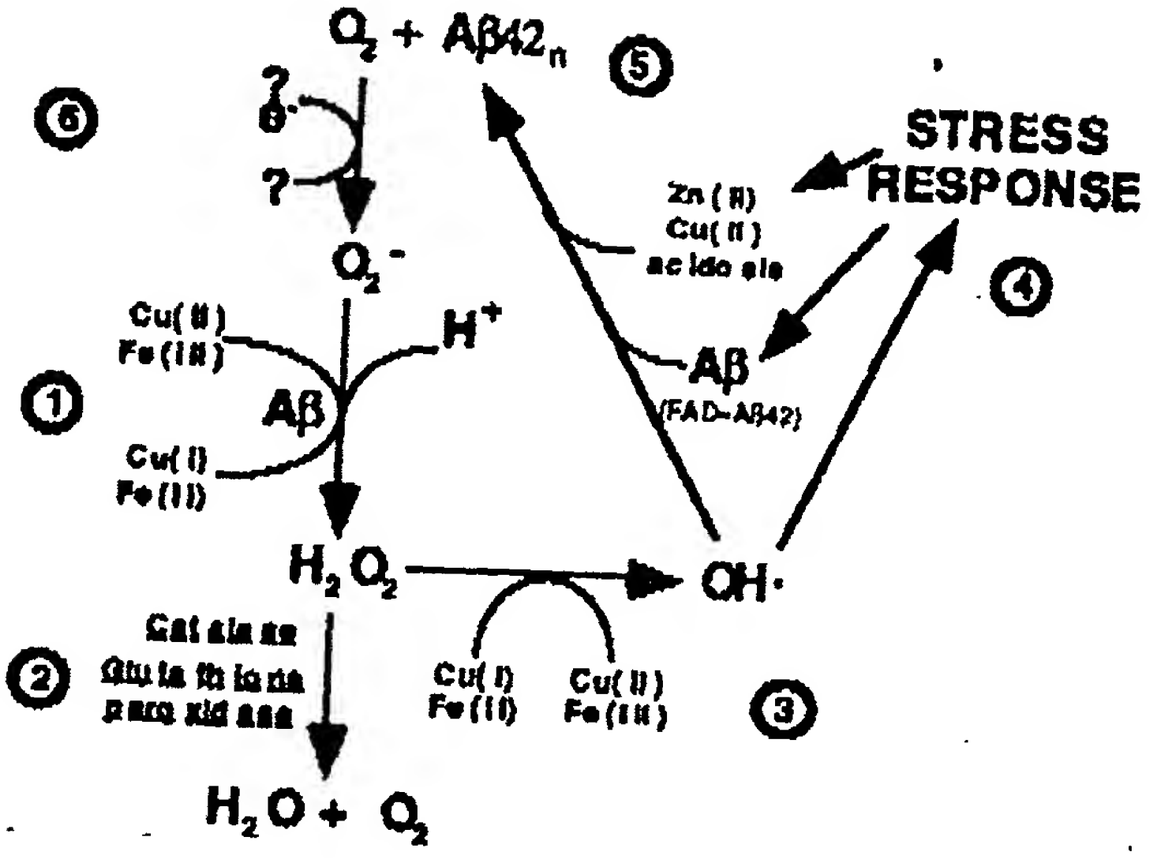


Fig. 12